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Validation and Functional Analysis of Ovine Herpesvirus 2-encoded microRNAs

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Declaration

I declare that this thesis is of my own composition, and that it contains no material previously submitted for the award of any other degree. The work described in this thesis has been executed by myself, all work of other authors is duly acknowledged.

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Abstract

Ovine herpesvirus 2 (OvHV-2) is a gammaherpesvirus of domestic sheep and causes the lymphoproliferative disease malignant catarrhal fever (MCF) in susceptible ruminants, including cattle. Sheep are latently infected but do not develop disease. MCF is characterised by proliferation of non-antigen specific cytotoxic large granular lymphocytes which leads to necrosis of infiltrated tissues and death. The molecular basis underlying MCF pathogenesis is poorly understood and it is unknown what controls the differences in the clinical outcome of infection between sheep and cattle, two closely related species.

microRNAs (miRNAs) are short noncoding RNAs that post-transcriptionally regulate gene expression through targeting of mRNA. A number of herpesviruses have been shown to encode miRNAs that are capable of regulating of both viral and cellular gene expression which can often have an effect on the pathogenesis of the virus. Following RNA seq analysis of an OvHV-2-infected bovine T-cell line (BJ1035) forty-five miRNAs were predicted to be encoded. Eight miRNAs were previously validated by northern blotting, and a further twenty-seven were confirmed using two PCR methods described in this project.

It was hypothesised that these virus-encoded miRNAs may differentially target cellular genes in sheep and MCF-susceptible species. Previous work using the technique CLASH (Crosslinking Ligation and Sequencing of Hybrids) identified Delta-like 1 (*DLL1*), a ligand for Notch signalling, as a potential target of ovhv2-miR-17-2. Initially, differential targeting of *DLL1* between sheep and cattle was hypothesised due to differences in the sequence and number of binding sites for ovhv2-miR-17-2. The sheep *DLL1* mRNA was shown to be targeted however, due to incorrect annotation of the sheep genome, targeting of *DLL1* is likely in both sheep and cattle. One OvHV-2-encoded miRNA, ovhv2-miR-73-1, has partial homology to a mammalian miRNA, miR-216a. Based on this homology it was predicted that ovhv2-miR-73-1 may target Phosphatase and Tensin Homolog (*PTEN*) and Y Box Binding Protein 1 (*YB-1*), as they are known targets of miR-216a. A GFP-reporter system was used to demonstrate that despite having similar seed sequences, ovhv2-miR-73-1 does not target *PTEN* or *YB-1*. Bioinformatic prediction was used to identify MHC class II genes as potential targets of OvHV-2-encoded miRNAs. Two miRNAs, ovhv2-miR-17-25 and ovhv2-miR-17-9 were shown to target sheep MHC class II genes (*DRA* and *DQB* respectively) using a luciferase reporter system. These miRNAs were not predicted to target the equivalent genes in cattle indicating that these genes may be differentially regulated between sheep and cattle.

It was also shown that two OvHV-2-encoded miRNAs, ovhv2-miR-17-10 and ovhv2-miR-61-1, target the viral protein Ov2. Ov2 is predicted to contain a basic leucine zipper (bZIP) domain

and is therefore likely to be a transcription factor. Other closely related gammaherpesviruses encode proteins that contain bZIP domains and these play major roles in the reactivation of the virus from latency. Immunofluorescence and confocal microscopy was performed to confirm the nuclear localisation of Ov2. RT-qPCRs were performed to investigate whether Ov2 could regulate the expression of any cellular genes. Of the two genes investigated, one of these, Jagged (*JAG1*), was downregulated in the presence of an Ov2-EGFPN1 construct compared to a control plasmid. *JAG1* is another ligand for Notch signalling indicating that the virus may manipulate Notch signalling using multiple methods. Immunoprecipitation and mass spectrometry analysis of an Ov2HA-pcDNA3.1+ construct was performed and a number of potential interacting partners of Ov2 were identified.

Lay Summary

Malignant catarrhal fever (MCF) is a fatal disease of cattle, bison and deer caused by infection with the virus Ovine herpesvirus 2 (OvHV-2). Sheep are infected from a very young age and carry the infection for life without any signs of disease. This virus can spread to other sheep and also to cattle, bison and deer; if these "foreign" hosts catch the virus they develop MCF. This disease is economically important not only in the UK but also in Sub-Saharan Africa and other areas of the developing world where it places a major burden on food production. OvHV-2 infects and persists in particular types of cells in the immune system. In sheep, these cells continue to function normally however in cattle these same immune cells change and as a result are able to attack and kill other cells in the body resulting in disease and ultimately death. Given that sheep and cattle are very closely related species in terms of their genetics, one of the major questions in OvHV-2 biology is why sheep survive and cattle die. Herpesviruses are wide-spread throughout nature and usually only infect one species and have co-existed and evolved with that species for millions of years.

Messenger RNA (mRNA) carries the message or "blueprint" of the DNA to be able to make the protein. Previous work in the group has predicted that a number of molecules, termed microRNAs (miRNAs), would be produced by the virus. miRNAs work by affecting how much of a particular protein is produced in a cell by binding to the mRNA or blueprint for a particular protein and stopping its production. The hypothesis is that the OvHV-2 miRNAs have evolved to work in sheep immune cells, allowing the virus to persist, and that these miRNAs encoded by OvHV-2 may not do the same thing in a "foreign" host such as cattle.

The first aim of this project was to validate the predicted virus miRNAs and it was found that OvHV-2 produces at least thirty-five miRNAs. The next objectives were to find out what some of the targets of these miRNAs are in sheep and cattle. Computer prediction programmes were used to predict targets in sheep and cattle and experiments to confirm these interactions were performed. Two targets were confirmed in sheep and cattle and both of these play a role in the immune response to infection. One of these is potentially targeted differently between sheep and cattle indicating that this may influence the outcome of disease in the closely related species. Another confirmed target was a virus protein that is thought to be important in interacting with the host, although its exact function is unknown. Experiments are being continued by others in the group to find out the function of this virus protein and how it relates to the development of disease.

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Abbreviations

3'	3 prime
5'	5 prime
~	approximately
$	dollar
%	percent
*	asterisk
°C	degrees Celsius
α	alpha
β	beta
γ	gamma
δ	delta
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
Ago	Argonaute
AIDS	acquired immune deficiency syndrome
AlHV-1	alcelaphine herpesvirus 1
AlHV-2	alcelaphine herpesvirus 2
AP-1	activator protein 1
APC	antigen presenting cell
ATF	actvating transcription factor
BALF	BamHI A leftward reading frame of Epstein-Barr virus
BART	BamHI A rightward transcript of Epstein-Barr virus
Bcl-2	B cell lymphoma 2
BCL2L10	BCL2-Like 10
BCLAF1	Bcl-2 associated transcription factor 1
BcLF1	BamHI c leftward reading frame 1 of Epstein-Barr virus
BCPV	Bandicoot papillomatosis carcinomatosis virus
BCR	B cell receptor
BDLF1	BamHI D leftward reading frame 1 of Epstein-Barr virus
BdRF1	BamHI d rightward reading frame 3 of Epstein-Barr virus
BFRF3	BamHI F rightward reading frame 3 of Epstein-Barr virus
BHK	Baby Hamster Kidney
BHRF	Bam HI fragment H rightward open reading frame of Epstein-Barr virus
BLAST	Basic local alignment search tool
BLASTN	Basic local alignment search tool of nucleic acids
BLAT	BLAST-like Alignment Tool
BLV	bovine leukaemia virus
BMLF1	BamHI-M leftward reading frame 1 of Epstein-Barr virus
BMRF2	BamHI M leftward reading frame 2 of Epstein-Barr virus
BORF	BamHI O rightward reading frame of Epstein-Barr virus
bp	base pair

BRLF1	BamHI R leftward reading frame 1 of Epstein-Barr virus
BSA	bovine serum albumin
BSLF1	BamHI S leftward reading frame 1 of Epstein-Barr virus
BVRF2	BamHI V rightward reading frame 3 of Epstein-Barr virus
BXRF1	BamHI X rightward reading frame of Epstein-Barr virus
bZIP	basic leucine zipper domain
BZLF1	BamHI Z leftward reading frame 1 of Epstein-Barr virus
CASP3	caspase-3
CASP8	caspase-8
CCNE2	cyclin E2
CD	cluster of differentiation
CDK2	cyclin-dependent kinase 2
cDNA	complementary deoxyribonucleic acid
CIITA	class II MHC transactivator
CLASH	cross linking ligation and sequencing of hybrids
cm	centimetre
CO2	carbon dioxide
CpHV-2	caprine herpesvirus 2
CR2	complement receptor 2
CXCL	chemokine (C-X-C motif) ligand
DAPI	4',6-diamidino-2-phenylindole
DC-SIGN	Dendritic cell specific intracellular adhesion molecule-3 grabbing non-integrin
DENND2D	DENN/MADD Domain Containing 2D
DGCR8	DiGeorge syndrome critical region gene 8
DLL1	delta-like 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPI	days post infection
ds	double stranded
E	early
EBER	Epstein-Barr virus encoded ribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen leader protein
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EHV-2	equine herpesvirus 2
eIF	eukaryotic translation initiation factor
EphA2	ephrin receptor A2
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FADD	Fas-associated death domain protein
FBS	foetal bovine serum

FC	flow cytometry
FLICE	Fas-associated death domain protein-like interleukin-1 beta-converting enzyme
FRA	FOS-related antigen
GaHV-2	Gallid herpesvirus 2
GATA3	GATA Binding Protein 3
gDNA	genomic DNA
GDP	Guanosine diphosphate
GFP	green fluorescent protein
gp	glycoprotein
GTP	Guanosine triphosphate
GZMA	granzyme A
H_20	Water
HAT	histone acetyltransferases
HCl	hydrochloric acid
HCMV	human cytomegalovirus
HDAC	histone deacteylases
HEK-293T	human embryonic kidney cells
HHV	human herpesvirus
HITS-CLIP	high throughput sequencing of RNAs isolated by UV crosslinking and immunoprecipitation
HIV	human immunodeficiency virus
HPV	human papillomavirus
hrs	hours
HS	heparan sulfate
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
HVS	herpesvirus saimiri
ICAM-3	intracellular adhesion molecule-3
ICP	infected cell polypeptide
IE	immediate early
IF	immunofluorescence
IFNγ	interferon gamma
IgG	immunoglobulin
IL	interleukin
ILTV	infectious laryngotracheitis virus
IM	infectious mononucleosis
IP	immunoprecipitation
IRAK1	interleukin-1 receptor-associated kinase 1
JAG1	jagged 1
JCV	human polyoma JC virus
kb	kilobase
kbp	kilobase pairs
kDa	kilodalton

km	kilometre
KSHV	Kaposi's sarcoma-associated herpesvirus
L	late
LANA	latency associated nuclear antigen
LARII	luciferase assay reagent II
LAT	latency associated transcripts
LB	Luria Bertani
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LGL	large granular lymphocyte
LMP	latent membrane protein
LTR	left terminal repeat
M	molar
MAF	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog
MAPK	mitogen activated protein kinase
MCF	malignant catarrhal fever
MCFV	Malignant catarrhal fever virus
MCFV-WTD	Malignant catarrhal fever virus - White tailed deer
MCMV	murine cytomegalovirus
MDBK	Madin-Darby bovine kidney cells
MDV	Marek's disease virus
mfe	minimum free energy
MFI	medium fluorescence intensity
$MgCL_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MHC	major histocompatibility complex
MHV-4	murid herpesvirus 4
MHV-68	murine gammaherpesvirus 68
MICB	MHC Class I Polypeptide-Related Sequence B
MID	middle
min	minute
miRNA	microRNA
MK2	mitogen activated protein kinase activated protein kinase 2
ml	millilitre
mM	millimolar
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein complexes
MTA	messenger RNA transport and accumulation protein
Myd88	Myeloid differentiation primary response gene 88
NaB	sodium butyrate
NaCl	sodium chloride
NCBI	National centre for biotechnology information
NFATC2	Nuclear Factor Of Activated T-Cells, Cytoplasmic, Calcineurin-Dependent 2
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells

ng	nanogram
NGS	normal goat serum
NICD	notch intracellular domain
NK	natural killer
NKG2D	natural-killer group 2, member D
nM	nanomolar
nm	nanometre
NME	nucleoside diphosphate kinase
NP40	Nonidet P-40
NPC	nasopharyngeal carcinoma
nt	nucleotide
ORF	open reading frame
OvHV-2	ovine herpesvirus 2
p38	protein 38
p53	protein 53
PACT	protein activator of PKR
PAR-CLIP	photoactivable-ribonucleoside-enhanced crosslinking and immunoprecipitation
PAZ	Piwi Argonaut and Zwille domain
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with tween 20
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate
PIWI	P-element-induced wimpy testes
PKR	protein kinase R
pmol	picomole
PMSF	phenylmethanesulfonylfluoride
polII	RNA polymerase II
pre-miRNA	precursor microRNA
PRF1	perforin
pri-miRNA	primary microRNA
pSILAC	pulsed stable isotope labelling by amino acids in cell culture
PTEN	phosphatase and tensin homologue
PUMA	p53-up-regulated modulator of apoptosis
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
RANTES	regulated on activation, normal T cell expressed and secreted
Rb	retinoblastoma
Rbl2	retinoblastoma-Like 2
RBP-J	J kappa-recombination signal binding protein
RISC	RNA-induced silencing complex
RL	repeat sequence long

RLC	RISC loading complex
RNA	ribonucleic acid
rpm	revolutions per minute
RS	repeat sequence short
RT	reverse transcription
RTA	replication and transcription activator
RTR	right terminal repeat
SA-MCF	sheep associated malignant catarrhal fever
SDS-PAGE	Sodium dodecyl sulfate poly acrylamide gel electrophoresis
SEF	sheep embryonic fibroblast cells
siRNA	small interfering RNA
SMAD	Sma/mothers against decapentaplegic homologue
SOC	Super optimal broth with catabolite repression
SOX	ShutOff and Exonuclease protein of KSHV
SRrp53	SR-related protein of 53 kDa
SRSF1	Serine/Arginine-Rich Splicing Factor 1
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBRP	TAR RNA-binding protein
TBS-T	Tris-buffered saline with Tween
TGF-β	Transforming growth factor beta
THBS1	thrombospondin 1
TNFSF12	tumour necrosis factor ligand superfamily member 12
TNFα	tumour necrosis factor alpha
TPA	tetradecanoylphorbol acetate
TSC22D1	TSC22 Domain Family, Member 1
TWEAKR	tumour necrosis factor receptor superfamily member 12A
U	unit
UL	unique long
US	unique short
UTR	untranslated region
UV	ultraviolet
V	volts
v/v	volume per volume
v-FLIP	viral FLICE-inhibitory protein
vIRF3	viral interferon regulatory factor-3
VZV	varicella zoster virus
w/v	weight per volume
WA-MCF	wildebeest associated malignant catarrhal fever
WB	western blotting
WC1	workshop cluster 1
YB-1	Y box binding protein 1
ZEB1	zinc finger E-box-binding homeobox 1

Chapter 1: Introduction

1.1 Herpesviruses

Herpesviruses are large, double stranded (ds) DNA viruses that are widely distributed in nature. Most animal species are thought to be infected with at least one herpesvirus (Pellet & Roizman, 2007). Over two hundred herpesviruses have been isolated so far, and it is likely that many more exist but have not yet been discovered due to lack of research in a number of animal species (Wibbelt *et al.*, 2007). Herpesviruses are thought to have co-evolved closely with their hosts for millions of years. This is supported by evidence that shows sequence divergence but conserved function of open reading frame (ORF) homologues between different herpesviruses (Fossum *et al.*, 2009).

Herpesviruses can cause a broad range of clinical outcomes from an asymptomatic infection through to death. Fatal conditions caused by herpesvirus infection such as encephalitis and tumour formation are usually associated with immunocompromised hosts or infection in a non-natural host.

The *Herpesviridae* family share a number of biological properties. First, herpesviruses encode a large number of enzymes involved in DNA synthesis, nucleic acid metabolism and protein processing. Second, herpesvirus genome replication and capsid assembly occurs in the nucleus. Third, herpesviruses are capable of maintaining a lifelong infection (latent infection) in their natural host. During latency, the virus genome is present within the cell as an episome and a small subset of viral genes are expressed. Latent virus is able to reactivate following a stress event where full gene expression and production of infectious virions occur (Pellet & Roizman, 2007).

1.1.1 Herpesvirus Structure

The structure of herpesviruses is well conserved. A typical herpesvirus virion is shown in Figure 1.1. The core of the particle contains the linear ds genome of the virus tightly packed within an icosahedral capsid of T = 16 symmetry. The approximate size of the capsid is between 100 and 110 nm in diameter. The capsid is surrounded by the tegument; a loosely organised protein structure. It is thought that proteins in the tegument play important roles in initiating infection, regulation of virus gene expression and virus assembly (Penkert & Kalejta, 2011; Yu *et al.*, 2011). A lipid envelope derived from the infected cell surrounds the tegument. Embedded within the envelope are a number of viral glycoproteins which play a role in virus entry (discussed further in section 1.3.1). The size of the virion including tegument and envelop can range from 120 nm to 300 nm due to variation in the thickness of the tegument (Pellet & Roizman, 2007).

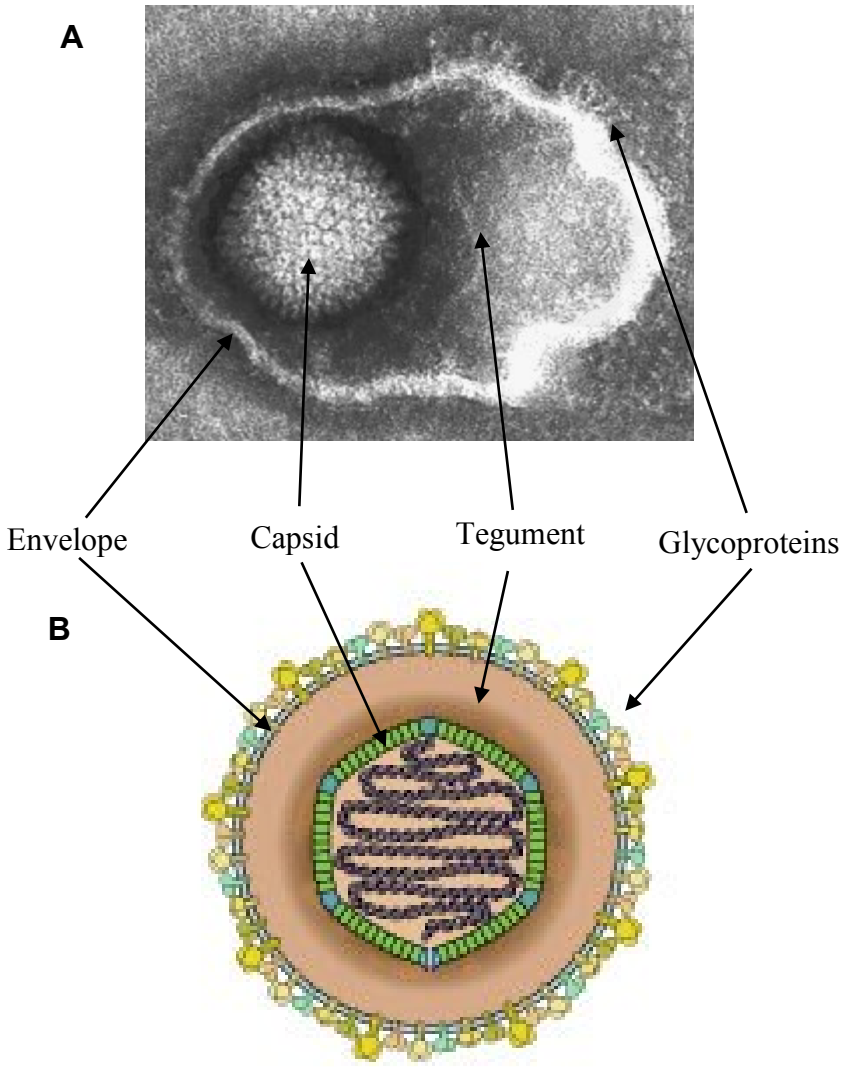


Figure 1.1 A typical herpesvirus virion

A: An electron micrograph of herpes simplex virus 1 (taken from http://web.stanford.edu/group/virus/herpes/2000/herpes2000.html).

B: A diagrammatical representation showing the major structural features envelope, capsid, tegument and glycoproteins (adapted from http://viralzone.expasy.org/all_by_protein/987.html)

1.1.2 Herpesvirus Genomes

The genomes of herpesviruses range in length from approximately 120 to 245 kilobase pairs (kbp) and encode at least 70 ORFs (McGeoch *et al.*, 2006). The upper limit of number of ORFs encoded by herpesviruses is not defined. Human cytomegalovirus is estimated to encode between 164 and 252 ORFs although recent evidence has shown that there may be as many as 751 ORFs translated (Davison *et al.*, 2003; Murphy *et al.*, 2003; Stern-Ginossar *et al.*, 2012). A minimum of 43 ORFs are thought to be conserved across the herpesvirus family; these core genes code for proteins involved in DNA replication as well as for capsid and tegument proteins (McGeoch & Davison, 1999). The remaining ORFs may be conserved within herpesvirus subfamilies (alpha, beta and gamma) or may be unique to the herpesvirus itself. In addition many herpesviruses have one or more non-coding regions of the genome which do not contain any ORFs but may code for non-coding RNAs (Chandriani *et al.*, 2010; Hart *et al.*, 2007). Several herpesviruses have been shown to code for microRNAs (miRNAs), and this will be discussed further in section 1.7 (Kincaid & Sullivan, 2012).

The unique sequence of herpesvirus DNA is often flanked by inverted or terminal repeat (reiterated) sequences. The copy number of these terminal repeat sequences can affect the length of the genome of any given herpesvirus. This explains why herpesvirus genome length is often written as an approximation rather than an exact value. Herpesviruses can be categorised into six groups A – F depending on the presence, location and number of the repeat sequences (Roizmann *et al.*, 1992). Figure 1.2 shows a diagrammatic representation of the six groups based on their repeat sequence architecture. Group A viruses have a large region from one terminus of the genome directly repeated at the other terminus. These regions are designated the left terminal repeat (LTR) and right terminal repeat (RTR). In group B viruses, a variable number of directly repeated sequences are present at both ends of the genome. Group C viruses also contain a variable number of directly repeated sequences at both ends of the genome however these are often fewer in number compared to group B viruses. Group C viruses also contain unrelated, directly repeated sequences greater than 100 base pairs (bp) that subdivide the genome. In group D viruses, the unique sequence is divided into unique long (U_L) and unique short (U_S) regions. The U_S region is separated from the U_L region by an inverted repeat of the sequence at the other terminus of the U_S region. Similarly the genomes of group E viruses are divided into U_L and U_S regions. Both the U_L and U_S regions are flanked by their own inverted repeat sequence (designated R_L or R_S). The genomes of group F viruses have not been found to contain any repeat sequences (Roizmann *et al.*, 1992).

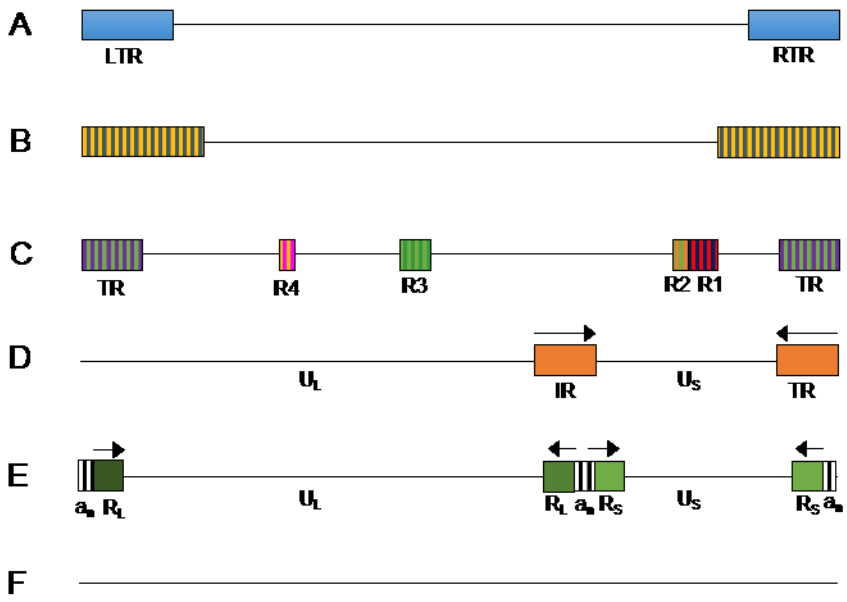


Figure 1.2 Schematic diagram of the sequence arrangement of the 6 classes of viral genomes in the *Herpesviridae* family.

Genomes are represented by lines and repeat sequences by rectangles. Relative orientations of repeat sequences are indicated by arrows. The genomes of group A viruses (e.g. channel catfish herpesvirus) contain a large sequence from one terminus directly repeated at the other terminus. Group B viruses (e.g. *Saimiriine herpesvirus 2*) have genomes which contain a variable number of directly repeated sequences at both termini. The genomes of group C viruses (e.g. Epstein-Barr virus) contain both terminal repeats and other unrelated repeat sequences that subdivide the genome. The unique regions of the genomes of group D viruses (e.g. varicella-zoster virus) are divided into two segments separated by an inverted repeat of the terminal region from the short (U_s) region. The unique regions of the genomes of group E viruses (e.g. herpes simplex virus and human cytomegalovirus) are similarly divided with inverted repeats flanking both the long (U_L) and U_s regions. No repeat regions have been described in the genomes of group F viruses (e.g. tupaia herpesvirus). Adapted from Roizmann *et al*., 1992.

1.2 Herpesvirus Classification

The *Herpesviridae* family is divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Classification of herpesviruses into the three subfamilies used to be based on biological characteristics such as host range and cell type in which a latent infection was established. Classification of herpesviruses is now based on DNA and protein sequence homology and similarities in genome arrangement (Roizmann *et al.*, 1992). This has resulted in the re-classification of Marek's disease virus (MDV) as an alphaherpesvirus rather than a gammaherpesvirus.

1.2.1 Alphaherpesviruses

The *Alphaherpesvirinae* subfamily is divided into 4 genera: *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus*. The *Simplexvirus* genus includes herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2). The *Varicellovirus* genus include varicella-zoster virus (VZV). The *Mardivirus* genus includes MDV and the *Iltovirus* genus includes infectious laryngotracheitis virus (ILTV). Alphaherpesviruses can productively infect a broad range of cell types however latency is primarily established in neuronal cells.

HSV-1 (human herpesvirus 1, HHV-1) and HSV-2 (human herpesvirus 2, HHV-2) were the first of the human herpesviruses discovered, and as the naming of the viruses suggests are closely related. HSV-1 and HSV-2 are widespread and are associated with oral and genital infections respectively. Outcomes of HSV infection can range from mild cutaneous lesions to encephalitis which can often be fatal. Primary HSV infection is usually asymptomatic and latency is established in the sensory ganglia innervating the site of infection. Reactivation of HSV-1 from latency is associated with disease; symptoms (typically cold sores) include small, grouped vesicles or blisters on epithelial surfaces. These then pustulate, ulcerate and later form a crust. Other conditions associated with HSV-1 infection include eczema, conjunctivitis leading to vision loss, erythema multiforme and fatal encephalitis. Transmission of HSV-2 from mother to foetus during pregnancy occurs rarely but can also cause encephalitis (Whitley & Roizman, 2001).

VZV (human herpesvirus 3, HHV-3) is the aetiological agent of chicken pox (also known as varicella), characterised by widespread skin lesions. VZV, like HSV-1 and HSV-2 is also widespread throughout the population. It is highly transmissible with primary infection occurring during childhood via skin to skin contact and inhalation of infected droplets. Primary infection with VZV can also be associated with a severe pneumonia, minor abnormalities in liver function, encephalitis and cerebellar ataxia (Moffat *et al.*, 2007). VZV establishes latency in sensory ganglia, and, in contrast to HSV infections, reactivation usually occurs only once

unless an individual is immunocompromised (Kennedy *et al.*, 1998). Reactivation of VZV results in herpes zoster or shingles, characterised by a painful, unilateral vesicular rash restricted to a single dermatome. Post-herpetic neuralgia can occur after resolution of the herpes zoster rash.

MDV (gallid herpesvirus 2, GaHV-2) is the causative agent of Marek's disease in chickens and is associated with T cell lymphoma. Due to the oncogenic potential of MDV it was originally classified as a gammaherpesvirus but was reclassified as an alphaherpesvirus based on its sequence homology and gene organisation (Buckmaster *et al.*, 1988). MDV primary infection occurs in B lymphocytes and macrophages in the lungs following inhalation of virus particles in contaminated dust. Latency is established in T cells (mainly CD4+), some of which will then undergo transformation resulting in lymphoma. This is different from other alphaherpesviruses described which only enter latency in neuronal cells (Couteaudier & Denesvre, 2014). Vaccines for MDV exist which exhibit a strong ability to control the disease however they do not protect against MDV infection (Haq *et al.*, 2013).

1.2.2 Betaherpesviruses

The *Betaheresvirinae* subfamily is divided into 4 genera: *Cytomegalovirus* (including human cytomegalovirus, HCMV), *Muromegalovirus* (including murine cytomegalovirus, MCMV), *Roseolovirus* (including human herpesvirus 6, HHV-6 and human herpesvirus 7, HHV-7) and *Proboscivirus* (including elephant endotheliotropic herpesvirus). Betaherpesviruses typically have a narrower host range than that of alphaherpesviruses and have a long replication cycle. Productive infection of betaherpesviruses occurs in a wide range of cell types within the host however latency of HCMV is thought to establish in CD34+ cells and cells of the myeloid lineage (Hanley & Bollard, 2014)

HCMV (human herpesvirus 5, HHV-5) is a leading cause of congenital infections worldwide. Congenital HCMV infection is associated with hearing loss, mental retardation and seizures in neonates (Pass *et al.*, 2006). It is also of major concern in immunocompromised individuals, such as transplant patients and human immunodeficiency virus (HIV) infected individuals where HCMV-associated pneumonia can be potentially fatal (Gandhi *et al.*, 2003). Primary HCMV infection (in an immunocompetent host) is usually asymptomatic although it can result in an infectious mononucleosis (IM, similar to that caused by Epstein-Barr virus, EBV). Latency establishment and maintenance is poorly understood in HCMV. Reactivation of the virus can occur in response to immunosuppression or stress however the mechanism has not yet been elucidated (Crough & Khanna, 2009).

1.2.3 Gammaherpesviruses

The *Gammaherpesvirinae* subfamily was traditionally divided into 2 genera: *Lymphocryptovirus* (including EBV) and *Rhadinovirus* (including Kaposi's sarcoma-associated herpesvirus, KSHV, and murine gammaherpesvirus 68, MHV-68). The *Herpesviridae* Study Group of the International Committee on Taxonomy of Viruses has increased the number of genera from two to four to include *Macavirus* (including viruses associated with malignant catarrhal fever, MCF, see section 1.4 for details) and *Percavirus* (including equine herpesvirus 2) (Davison *et al.*, 2009; McGeoch *et al.*, 2006). Gammaherpesviruses are typically thought to have a narrow host range, however this can sometimes extend across the order or family to which the natural host belongs. Unlike alphaherpesviruses and betaherpesviruses, lytic replication of gammaherpesviruses occurs in a small subset of cells. In addition, gammaherpesviruses establish and maintain latency in lymphocytes, usually either B lymphocytes or T lymphocytes. Gammaherpesviruses also tend to encode unique genes not conserved across the *Herpesviridae* family. These are usually located towards the termini of the genome, can be homologues of cellular genes and often play roles in viral pathogenesis.

EBV (human herpesvirus 4, HHV-4) is the best studied gammaherpesvirus. EBV is highly prevalent and is thought to infect over 90% of the human population worldwide. Transmission of EBV is thought to be by the oral route, however infection can also occur through blood transfusion and organ donation. Primary infection usually occurs in childhood and is asymptomatic, however if primary infection does not occur until adolescence IM can develop (Chen, 2011). Symptoms of IM include fever, pharyngitis, lymphadenopathy, malaise and fatigue (Rea *et al.*, 2001). Latency is established in B lymphocytes and is associated with a number of malignancies including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma (NPC), primary effusion lymphoma and post-transplant lymphoproliferative disease (Ambinder & Cesarman, 2007). Although EBV-associated malignancies are associated with the immunocompromised, immunocompetent individuals may develop EBV-associated cancers due to the genetic background of the host or environmental factors. Latency of EBV will be discussed further in section 1.3.3.

KSHV (human herpesvirus 8, HHV-8) is the most recently discovered human herpesvirus. Like EBV, KSHV is associated with a number of malignancies including Kaposi's sarcoma (KS), multicentric Castleman's disease and primary effusion lymphoma. KSHV is endemic in sub-Saharan Africa and regions surrounding the Mediterranean. KS was historically seen in elderly Mediterranean men and presents with a small number of cutaneous lesions on the lower

legs (classic KS). A rise in the incidence of KS occurred in the 1980s as a result of the HIV/AIDS (acquired immunodeficiency syndrome) epidemic. Furthermore, KS is the most common cancer diagnosed in AIDS patients in sub-Saharan Africa (Mesri *et al.*, 2010). AIDS-associated KS is a much more aggressive cancer than classic KS; lesions are widespread and often form on the lining of internal organs. Primary infection most likely occurs at an early age from contact via saliva and latency is established and maintained primarily in peripheral B lymphocytes (Blackbourn *et al.*, 2000; Martro *et al.*, 2004). Latency of KSHV will be discussed further in section 1.3.3.

MHV-68 (murid herpesvirus 4, MHV-4) is a member of the *Rhadinovirus* genus and is a useful model for the study of gammaherpesvirus infection *in vivo* (Simas & Efstathiou, 1998; Sunil-Chandra *et al.*, 1992). As MHV-68 was originally isolated from the bank vole (*Cletrinomys glarioulus*) in Slovakia, the *in vivo* model of MHV-68 does not represent a natural infection (Blaskovic *et al.*, 1980). Transmission of MHV-68 occurs through the intranasal route and lytic infection takes place in the alveolar epithelial cells in the lung. Following this, virus dissemination occurs and latent infection of B lymphocytes takes place. Splenomegaly is a hallmark of latent infection as infected B lymphocytes reside in the spleen (Nash *et al.*, 2001). The splenomegaly resolves over the course of a few weeks and long term latent infection is maintained. Although the spleen is the major reservoir for latent MHV-68, the virus can be found latently infecting other tissues including the lung (Flaño *et al.*, 2003).

Saimiriine herpesvirus 2, (herpesvirus saimiri, HVS) is the prototypic *Rhadinovirus*. The natural host of HVS is the squirrel monkey (*Saimiri sciureus*). They become infected early in life through contact with saliva of other infected squirrel monkeys. HVS does not cause apparent disease in its natural host but is capable of causing lymphomas, particularly induction of T cell lymphoma, in other monkey species following experimental infection (Fickenscher & Fleckenstein, 2001). The immortalisation of human T lymphocytes in cell culture has been made possible because certain strains of HVS (e.g. C488) have the ability to transform human T lymphocytes without the need for re-stimulation by an antigen or mitogen (Fleckenstein & Ensser, 2001).

Viruses in the genus *Macavirus* include ovine herpesvirus 2 (OvHV-2) and alcelaphine herpesvirus-1 (AlHV-1) and have been shown to cause MCF in susceptible species (Russell *et al.*, 2009). These viruses will be discussed further in section 1.5.

1.3 Herpesvirus Lifecycle

Herpesviruses have two distinct stages in their host: the lytic stage and the latent stage. The lytic stage involves expression of the majority of virus-encoded ORFs followed by production and subsequent release of infectious virus particles. This is in contrast to the latent stage where gene expression is highly restricted and the viral genome persists in the infected cell as an episome. No infectious virus is produced during latency. It could be said that a third stage of the herpesvirus lifecycle exists which is reactivation from latency. Latent virus is able to reactivate following a number of stimuli such as immunosuppression or cellular stress. Full viral replication resumes and infectious virus is produced and released.

1.3.1 Attachment and Entry

The first stage in the herpesvirus lifecycle is attachment and entry. Attachment and entry (or fusion) are two different processes in herpesviruses often utilising different viral proteins and host receptors. Given that herpesviruses undergo lytic replication and establish and maintain latency in different cell types it is not surprising that the mechanism of attachment and entry is very complicated for herpesviruses. They code for a number of different glycoproteins that act as mediators of attachment and entry and can also utilise a number of different cellular receptors. Endocytosis or cell fusion are the most common methods of herpesvirus entry into a host cell (Shukla & Spear, 2001).

In EBV eight glycoproteins have been identified that play a role in virus entry of either B lymphocytes or epithelial cells (Hutt-Fletcher, 2007). Attachment of EBV in B lymphocytes involves the interaction of the EBV envelope protein gp350/220 and the cellular complement receptor type 2 (CR2) (Fingeroth *et al.*, 1984). Fusion of EBV with a B cell is mediated by endocytosis and requires the viral proteins gHgL, gB and gp42. gHgL and gB are conserved across herpesviruses and make up the core fusion machinery. gp42 is only conserved across the genus *Lymphocryptovirus* and together with gHgL acts as the trigger for fusion in B cells (Hutt-Fletcher, 2007; Rivailler *et al.*, 2002). In epithelial cells it is not known whether CR2 is expressed *in vivo* however there is low level expression of CR2 in some cultured cells. In the absence of CR2, gHgL can act as a ligand for attachment to an unknown cellular receptor (Molesworth *et al.*, 2000). Another candidate for attachment of EBV is the virus membrane protein BMRF2 which has been shown to interact with a number of integrins (Tugizov *et al.*, 2003; Xiao *et al.*, 2007). Fusion of EBV with epithelial cells does not appear to require endocytosis (Miller & Hutt-Fletcher, 1992). In contrast to fusion with B cells, gp42 is dispensable and its presence is in fact inhibitory (Wang *et al.*, 1998). gB is required for fusion with epithelial cells in higher amounts than for B cell penetration suggesting that gB is not

required for endocytosis but plays a large role in fusion (Neuhierl *et al.*, 2002). It also possible that gp350/220 can impede attachment and entry of EBV with epithelial cells; antibodies to gp350/220 which are capable of blocking B cell attachment enhance epithelial cell infection (Turk *et al.*, 2006).

KSHV encodes a number of viral glycoproteins, some of which are conserved across herpesviruses (gB, gH, gL, gM and gN) and some of which are unique (ORF4, gpK8.1A, gpK8.1B, K1, K14 and K15). The attachment of KSHV is mediated through interactions with the widely expressed cell surface proteoglycan heparan sulfate (HS). Several KSHV-encoded glycoproteins are known to interact with HS including gB, gpK8.1A and gH (Birkmann *et al.*, 2001; Hahn *et al.*, 2009; Wang *et al.*, 2003). As in EBV, integrins have also been implicated as entry receptors for KSHV, at least in adherent cells *in vitro* (Akula *et al.*, 2002). It is unlikely that the interaction of KSHV with integrins is sufficient to trigger fusion and that a more complex interplay of virus-host interactions is required (Veettil *et al.*, 2008). One of the cellular receptors identified for cellular fusion is the cystine transporter xCT. As xCT has not been detected in B cells it is most likely that this interaction plays a role in KSHV entry of cells supporting lytic infection such as endothelial cells (Kaleeba & Berger, 2006). Dendritic cell specific intracellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN) is a C-type lectin usually expressed on dendritic cells (DCs) but can also be expressed on macrophages and B lymphocytes. DC-SIGN has been shown to be a mediator of endocytosis for KSHV on DCs, macrophages and B lymphocytes (Rappocciolo *et al.*, 2008). A third entry receptor, ephrin receptor A2 (EphA2), has been identified for KSHV. gHgL binds to EphA2 and triggers EphA2 phosphorylation and endocytosis and therefore internalisation of the virus in epithelial and endothelial cells *in vitro* (Hahn *et al.*, 2012).

1.3.2 Lytic Replication

Herpesvirus gene expression occurs in a highly regulated temporal cascade. In HSV-1 infection, immediate early (IE) genes (also known as alpha or α genes) are most highly expressed 3 to 4 hrs post infection. IE gene expression is followed by early (E) gene expression (also known as beta or β genes), which are most highly expressed 5 to 7 hrs post infection. Lastly, late (L) genes (also known as gamma or γ genes) are most highly expressed around 12 hrs post infection (Honess & Roizman, 1974).

In EBV two transactivator proteins control the lytic replication programme; BZLF1 (also known as Zta) and BRLF1 (also known as replication and transcription activator, RTA). BZLF1 and BRLF1 have non-redundant roles and co-operation of these two transactivators is required for virus production (Feederle *et al.*, 2000). The homologues of BZLF1 and BRLF1

in KSHV are K8 (K-bZIP) and ORF50 respectively. Expression of ORF50 alone, but not K8 alone, is sufficient to trigger lytic replication and is therefore the major transactivator in KSHV (Wang *et al.*, 2004b). The role of K8 is less well defined than that of ORF50 but is thought to co-regulate the function and modulate the transcriptional activity of ORF50 (Izumiya *et al.*, 2003). Another IE gene expressed is the mRNA transport and accumulation protein (Mta). In EBV this is also designated BMLF1 and ORF57 in KSHV. The role of ORF57 in KSHV infection is well studied. It is essential for virus production and plays role in accumulation, stabilisation and export of viral mRNAs (Boyne *et al.*, 2010). BMLF1 is thought to play a similar role in EBV infection as ORF57 does in KSHV infection (Semmes *et al.*, 1998).

The products of the IE genes initiate transcription of the E genes. E genes are primarily involved in DNA replication. These include the DNA polymerase (EBV BALF-5, KSHV ORF9), single stranded DNA binding protein (EBV BALF-2, KSHV ORF6) and viral primase (EBV BSLF1, KSHV ORF56). Other E gene products are involved in processes such as nucleotide metabolism. These include thymidine kinase (EBV BXLF1, KSHV ORF21) and ribonucleotide reductase (EBV BORF2 and BORF1 KSHV ORF61 and ORF60) (Jenner *et al.*, 2001). Herpesvirus DNA replication is dependent upon sites that function as lytic origins of replication (*oriLyt*). The EBV genome has one *oriLyt* whereas the KSHV genome has two (AuCoin *et al.*, 2002; Hammerschmidt & Sugden, 1988). It is proposed that herpesvirus DNA replication initiates through a theta mechanism in a bidirectional manner from the origin(s) of replication. The predominant mode of herpesvirus DNA replication is rolling circle replication; the switch between theta replication and rolling circle replication is undefined. Rolling circle replication results in the productions of long head-to-tail concatemers of viral DNA (Boehmer & Nimonkar, 2003).

Finally, late genes are transcribed which code for the proteins necessary for assembly and egress of the virus. A complex of proteins are required for the assembly of the virus capsid. In EBV these proteins are BcLF1, BORF1, BDLF1, BFRF3, BdRF1 and BVRF2. BdRF1 acts as a scaffold for the other proteins (except for BVRF2) for a procapsid structure to form (Henson *et al.*, 2009). Concatemers of DNA are cleaved and packaged into the newly formed capsids. This process is tightly coupled; cleavage occurs once one virus genome has filled one capsid (Boehmer & Lehman, 1997). The function of BVRF2 is to cleave the BdRF1 scaffold once the viral DNA has been packaged to form a mature capsid structure (Henson *et al.*, 2009).

Following packaging of viral DNA into capsids, nuclear egress occurs. The first step in this process is envelopment of the capsid in the inner nuclear membrane through budding into the perinuclear space (Johnson & Baines, 2011). Fusion with the outer nuclear membrane results

in de-envelopment of the capsid. At this point the tegument surrounds the capsid and a second envelope is acquired by budding through the trans-Golgi network. This results in transportation of virus particles to the plasma membrane for release by exocytosis (Mettenleiter, 2002).

1.3.3 Latency

During latency the viral genome circularises and is present as episomes within infected cells. No replication of the viral genome occurs and gene expression is highly restricted. The level of gene expression varies between herpesviruses. In this section, HSV-1 latency, as well as EBV and KSHV latency, will also be discussed.

Gene expression during HSV-1 latency is restricted to the latency associated transcripts (LATs) (Rock *et al.*, 1987; Stevens *et al.*, 1987; Wagner & Bloom, 1997). The LAT region lies antisense to ICP0, a major transactivator of HSV-1 gene expression (Kent *et al.*, 2003). As such, the LATs are thought to repress ICP0 function and regulate latency/reactivation (Jones, 2013). No protein is thought to be produced by the LATs and instead a number of small non-coding RNAs are produced. Eight miRNAs have been identified that map to, or near to, the LATs (Umbach *et al.*, 2009). It has been shown that miRNA-H2 encoded by HSV-1 specifically targets ICP0 expression promoting latency and increasing neurovirulence (Jiang *et al.*, 2015; Pan *et al.*, 2014). The function of herpesvirus-encoded miRNAs is discussed further in section 1.7. The LATs have anti-apoptotic effects and immune evasion properties. Viruses with deletions in the LATs have been shown to increase the rate of apoptosis in infected cells *in vivo* (Perng *et al.*, 2000). There is also evidence that LAT-deletion viruses show greater lytic gene expression implying that LATs down-regulate virus gene expression contributing to neurovirulence and survival (Garber *et al.*, 1997). This function also contributes to the immune evasion properties of LATs. Activation of the immune system could lead to a decrease in the number of latently infected cells (Chen *et al.*, 2000).

The latent state of KSHV is thought to be critical in the formation of tumours. During KSHV latency, at least six proteins are known to be expressed. These are the latency associated nuclear antigen (LANA, ORF73), v-cyclin (ORF72), v-FLIP (K13, ORF71) and the Kaposin transcripts A, B and C (K12) (Ganem, 2006). LANA is considered to play a major factor in the maintenance of latency. One of the main functions of LANA is to bind to the terminal repeat region of the viral genome and associate with nucleosomal proteins in order to tether the viral genome to the host genome thus maintaining viral episomes in daughter cells during cell division (Uppal *et al.*, 2014). LANA is, however, a multifunctional protein capable of the perturbation of many cellular pathways contributing to oncogenesis. Functions include

inhibiting the activity of tumour suppressors such as p53 and retinoblastoma (Rb) and promoting cell cycle progression (Wen & Damania, 2010). Two of the proteins listed above, v-cyclin and v-FLIP are homologues of cellular genes. v-cyclin is a homologue of cyclin D, a regulator of cell cycle progression. v-cyclin binds to, and activates, cyclin-dependent kinase 6, promoting cells to enter the S-phase of the cell cycle (Ganem, 2006). v-FLIP is a FLICE (FADD [Fas-associated death domain protein]-like interleukin-1 beta-converting enzyme) inhibitory protein. v-FLIP has anti-apoptostic functions, and is capable of blocking Fas-mediated apoptosis through abrogation of the interaction between FADD and caspase 8 (CASP8) (Wen & Damania, 2010). Kaposin A is encoded by ORF K12 and Kaposin B and C are expressed through differential initiation of translation; the start sites of translation of Kaposin B and C are upstream of Kaposin A (Ganem, 2006). Kaposin A has been shown to have oncogenic potential through interaction with cytohesin-1 and subsequent activation of the ERK (extracellular signal-regulated kinases) MAPK (mitogen activated protein kinase) signalling pathway (Kliche *et al.*, 2001). Kaposin B is known to activate the MAPK kinase 2 (MK2), which is usually a target of p38 phosphorylation. This results in the blocking of cytokine mRNA decay and enhancement of release of proinflammatory cytokines (McCormick & Ganem, 2006). A number of KSHV-encoded miRNAs are expressed during latency (Ramalingam *et al.*, 2012).

Like KSHV, latency in EBV is associated with tumorigenesis. There are three different programmes of latency in EBV infection, termed latency I, II and III which are defined by the viral transcripts present in the infected cell (Rowe *et al.*, 1992). The different states of latency are also associated with different populations of B cells and different cancers. Latency I is associated with memory B lymphocytes and Burkitt's lymphoma. Latency II is associated with induction of B lymphocyte differentiation and Hodgkin's lymphoma and NPC. Latency III is associated with activation of naïve B lymphocytes and post-transplantation lymphoproliferative diseases (Kang & Kieff, 2015; Odumade *et al.*, 2011). Two classes of protein – EBV nuclear antigen (EBNA) and latent membrane protein (LMP) are expressed along with EBV-encoded small RNAs (EBERs) and BART RNAs in latency. EBNA-1, LMP-2A/B, EBER1/2 RNA and BART RNA are detected in latency I. In latency II, the genes expressed include those in latency I and LMP-1. In latency III most of the latent genes are expressed; those expressed in latency I and II and five more EBNA proteins: EBNA-leader protein (EBNA-LP), EBNA-2, EBNA-3A, EBNA-EB and EBNA-3C (Kang & Kieff, 2015). As EBNA-1 is the homologue of LANA its functions will not be discussed further. The other EBNA proteins play roles in B lymphocyte transformation (EBNA-2, EBNA-3A and EBNA-3B) and perturbation of Notch signalling (Notch signalling is discussed further in section 4.3.1

and chapter 6), and have been shown to interact with p53 and Rb (EBNA-LP) and induce the expression of cellular CD40 (EBNA-3B) (Young & Murray, 2003). LMP-1 is often described as an oncogene in the literature and contributes to oncogenesis as a constitutively active CD40-like receptor inducing the expression of NF-κB (Pratt *et al.*, 2012). LMP-2A is thought to play a role in the maintenance of latency. It is thought that activation of the B cell receptor (BCR) is thought to disrupt latency; LMP-2A is capable of inhibiting BCR signalling and therefore preventing exit from latency (Masucci, 2004). LMP-2B, an amino-terminally truncated version of LMP-2A, has been shown to modulate LMP-2A activity through binding to and preventing phosphorylation of LMP-2A (Rovedo & Longnecker, 2007). The EBERs are proposed to play significant roles in EBV pathogenesis. They promote resistance to apoptosis by inhibiting protein kinase R (PKR) activation. EBER2 induces interleukin 6 (IL-6) expression contributing to B cell transformation (Iwakiri & Takada, 2010). It is now known that the BART RNAs produce a number of miRNAs (Pfeffer *et al.*, 2004).

1.3.4 Reactivation

Reactivation from latency is a highly controlled process. The mechanisms of reactivation for many herpesviruses have been elucidated however only some of the triggers have been identified. The outcome of reactivation is specific to different herpesviruses. Reactivation of alphaherpesviruses usually results in clinical symptoms such as cold sores and herpes zoster for HSV-1 and VZV respectively (Grinde, 2013). The reactivation of CMV is often controlled by the immune system in immunocompetent individuals however in the immunocompromised serious disease is often the outcome (Sinclair & Sissons, 2006). For the cancer-causing gammaherpesviruses EBV and KSHV, oncogenesis is usually associated with latency, however recent reports demonstrate an important role for subsets of infected cells undergoing lytic replication in the development of tumours (Cai *et al.*, 2010; Murata & Tsurumi, 2014).

Reactivation of KSHV from latency requires expression of the major transactivator ORF50. The triggers for reactivation are largely unknown however it has been shown that hypoxia may trigger the expression of ORF50. A number of chemical agents are also capable of reactivating the virus *in vitro* including sodium butyrate (NaB) and the protein kinase c inhibitor TPA (tetradecanoylphorbol acetate) (Cai *et al.*, 2010). Chromatin remodelling and epigenetic control of the KSHV genome have been shown to be critical in both the maintenance of and reactivation from latency. During latency, the KSHV genome undergoes chromatinisation and subsequent epigenetic modification to control gene expression (Lieberman, 2013). Tail modifications of histones play roles in regulation of chromatin structure and chromatin condensation, important factors in the regulation of transcription. The action of histone

acetyltransferases (HATs) leads to the loosening of chromatin increasing transcriptional availability; histone deacteylases (HDACs) have the opposite effect of tightening the chromatin structure leading to transcriptional inactivity (Niedermeier *et al.*, 2006). Furthermore, HDAC inhibitors have been shown to be able to reactivate KSHV from latency through remodelling of the promoter region of ORF50, which has been shown to be highly responsive to HDAC inhibitors (Lu *et al.*, 2003). DNA methylation (CpG dinucleotide methylation) is a method of epigenetic control of latency and reactivation. The ORF50 promoter has been shown to be heavily methylated during latency and demethylation of this region would be required for transcription of ORF50 and therefore subsequent reactivation (Chen *et al.*, 2001). Chromatin remodelling and epigenetic modification is not the whole story in KSHV reactivation; LANA, the major protein involved in the promotion of latency is also capable of repressing lytic gene expression (Lu *et al.*, 2006). It has been shown that inactivation of LANA through phosphorylation by cellular kinases Pim-1 and Pim-3 contributes to virus reactivation *in vitro* (Cheng *et al.*, 2009).

The triggers for reactivation of EBV also remain largely unknown, although it has been hypothesised that hypoxia can also lead to reactivation of EBV (Jiang *et al.*, 2006). Reactivation of EBV *in vitro* has been performed using a number of chemicals including NaB and TPA. Transforming growth factor beta (TGF-β) has also been shown to trigger EBV reactivation (Fahmi *et al.*, 2000). In contrast to KSHV reactivation where ORF50 is the major gene involved in reactivation, the EBV homologue BRLF1 is not thought to be important for EBV reactivation. Instead, BZLF1 is the major reactivation protein in EBV (Murata, 2014). Ectopic BZLF1 expression is sufficient to trigger EBV reactivation and subsequent lytic gene expression through binding of the *oriLyt* and acting as an adapter protein to recruit the viral replication machinery (Murata & Tsurumi, 2014; Schepers *et al.*, 1996). A cellular transcription factor, zinc finger E-box-binding homeobox 1 (ZEB1), can bind to the proximal promoter of BZLF1 and repress the transcription of BZLF1. A 2 base pair mutation in the silencer element of the promoter region was sufficient to reactivate EBV resulting in production of IE, E and L genes *in vitro*, indicating that ZEB1 plays a critical role in regulating the latent-lytic switch of EBV (Yu *et al.*, 2007). Activation of the cellular pathways Sma/mothers against decapentaplegic homologue (SMAD) and MAPK have been shown to be essential for EBV reactivation (Fahmi *et al.*, 2000; Liang *et al.*, 2002a). Both pathways are induced by expression of TGF-β possibly explaining why TGF-β is a trigger for EBV reactivation. Furthermore, BZLF1 also induces the expression of TGF-β creating a positive feedback loop initiating reactivation (Murata & Tsurumi, 2014). As in KSHV reactivation, EBV latency maintenance and reactivation requires chromatin remodelling and epigenetic

modifications (Lieberman, 2013). It has been demonstrated that high levels of histone acetylation is correlated with reactivation of EBV and that epigenetic silencing of the EBV genome occurs during latency (Countryman *et al.*, 2008; Ramasubramanyan *et al.*, 2012).

1.4 Malignant Catarrhal Fever

MCF is a frequently fatal lymphoproliferative disease that affects cattle and other ungulate species including deer, bison and pigs (Russell *et al.*, 2009). It is caused by viruses in the genus *Macavirus*, also known as the MCF virus (MCFV) group. Ten viruses have been identified so far that belong to the *Macavirus* genus (see Table 1.1 for a summary). Of these viruses, six (AlHV-1, AlHV-2, OvHV-2, CpHV-2, MCFV-WTD and Ibex-MCFV) have been associated with MCF in susceptible species. There have been no reported cases of MCF associated with the other four viruses, however it is possible that they are capable of causing disease in susceptible species (Li *et al.*, 2014). AlHV-1 and OvHV-2 are the main causative agents of MCF and will be discussed further in section 1.5.

1.4.1 Occurrence and Significance

MCF has a long history in veterinary medicine, South African farmers and Maasai pastoralists referred to the disease as snotziekte (mucous sickness). The association of MCF with wildebeest was made in the early 1920s (Mettam, 1924) . The association of MCF with sheep was made around 1930 when a number of cattle were able to be infected experimentally by being kept in close contact with sheep (Mushi & Rurangirwa, 1981). Wildebeest-associated MCF (WA-MCF) is a major problem in Africa and in zoological collections where susceptible species are housed closely with wildebeest (Heuschele, 1988; Meteyer *et al.*, 1989). Sheep-associated MCF (SA-MCF) is a problem worldwide as well as in zoological collections. Susceptibility to MCF varies widely between different species, SA-MCF can be a significant problem in highly susceptible species such as Bali cattle, farmed deer, Père David's deer and bison (Li *et al.*, 2014; Russell *et al.*, 2009). MCF has been documented in over thirty species including moose, reindeer, bongo (African forest antelope) and anoa (midget buffalo) (das Neves *et al.*, 2013; Gasper *et al.*, 2012; Li *et al.*, 2011b; Vikoren *et al.*, 2006).

The economic impact of MCF is unknown as there is no enforced reporting system for the disease. MCF is generally a sporadic disease with isolated cases although severe outbreaks can occur. In North America, outbreaks of MCF have forced bison producers out of business, with one case reporting loss of over eight hundred bison and financial losses of $1 million (Li *et al.*, 2006; O'Toole *et al.*, 2002).

Table 1.1 MCF viruses and their hosts

Virus	Natural Host	Susceptible Hosts
Alcelaphine herpesvirus 1	Wildebeest	Cattle Deer
Alcelaphine herpesvirus 2	Hartebeest Topi	Barbary red deer Bison
Hippotragine herpesvirus 1	Roan antelope	Not reported
Oryx-MCFV	Oryx	Not reported
Ovine Herpesvirus 2	Sheep	Cattle Deer Bison Pig Giraffe
Caprine herpesvirus 2	Goat	Sika deer
		White-tailed deer
MCFV-white tailed deer	Goat	White-tailed deer Red brocket deer Reindeer
Ibex-MCFV	Ibex	Bongo Anoa Pronghorn
Muskox-MCFV	Muskox	Not reported
Aoudad-MCFV	Aoudad	Not reported

1.4.2 Clinical Forms of MCF

There are five distinct clinical forms of MCF: head and eye, peracute, alimentary, neurological and cutaneous. The head and eye form is the most commonly reported form of MCF. The clinical course of infection varies depending on the susceptibility of the infected animal to MCF. In cattle, the incubation period is on average between two and ten weeks, however periods of up to nine months have been reported (O'Toole & Li, 2014). Typical signs of the head and eye form include pyrexia, depression, diarrhoea, ocular and nasal discharge and lesions of the buccal cavity and muzzle (Russell *et al.*, 2009). The peracute form of MCF is the most severe with death occurring in 24 to 72 hrs; bison are particularly affected by this form (O'Toole & Li, 2014). Symptoms often include depression, dysentery and diarrhoea (Holliman *et al.*, 2007). Symptoms of the alimentary form of MCF include ulceration in the gut and bloody, profuse diarrhoea (Brenner *et al.*, 2002). The cutaneous form is a rare form of MCF that presents with lesions on the base of the horns, dewclaws and the interdigital space (David *et al.*, 2005). Lethargy is often seen in the neurological form of MCF, as well as neck rigidity, incoordination and a stiff legged gait (Brenner *et al.*, 2002).

1.4.3 Pathology of MCF

Upon post mortem examination, gross findings include petechial haemorrhages on the tongue, buccal mucosa, gastrointestinal and respiratory tracts and urinary bladder (Figure 1.3). Raised pale foci are often seen on the kidneys and there is general lymph node enlargement (Russell *et al.*, 2009). Lymphoid accumulation is the main histological finding in a wide range of tissues. Lymphocytes and lymphoblasts are the main constituents of infiltration with fewer macrophages and monocytes present, however in severe lesions more macrophages than lymphocytes are present (Liggitt & DeMartini, 1980). Lymphoid accumulation in tissues is associated with degeneration and necrosis in many tissues, especially the epithelium. In general, virus particles are not found in lesions and it has been proposed that formation of lesions is due to lymphocyte dysregulation rather than direct antiviral responses, however one study has found evidence of OvHV-2 infected T lymphocytes in lesions (Li *et al.*, 2014; Simon *et al.*, 2003).

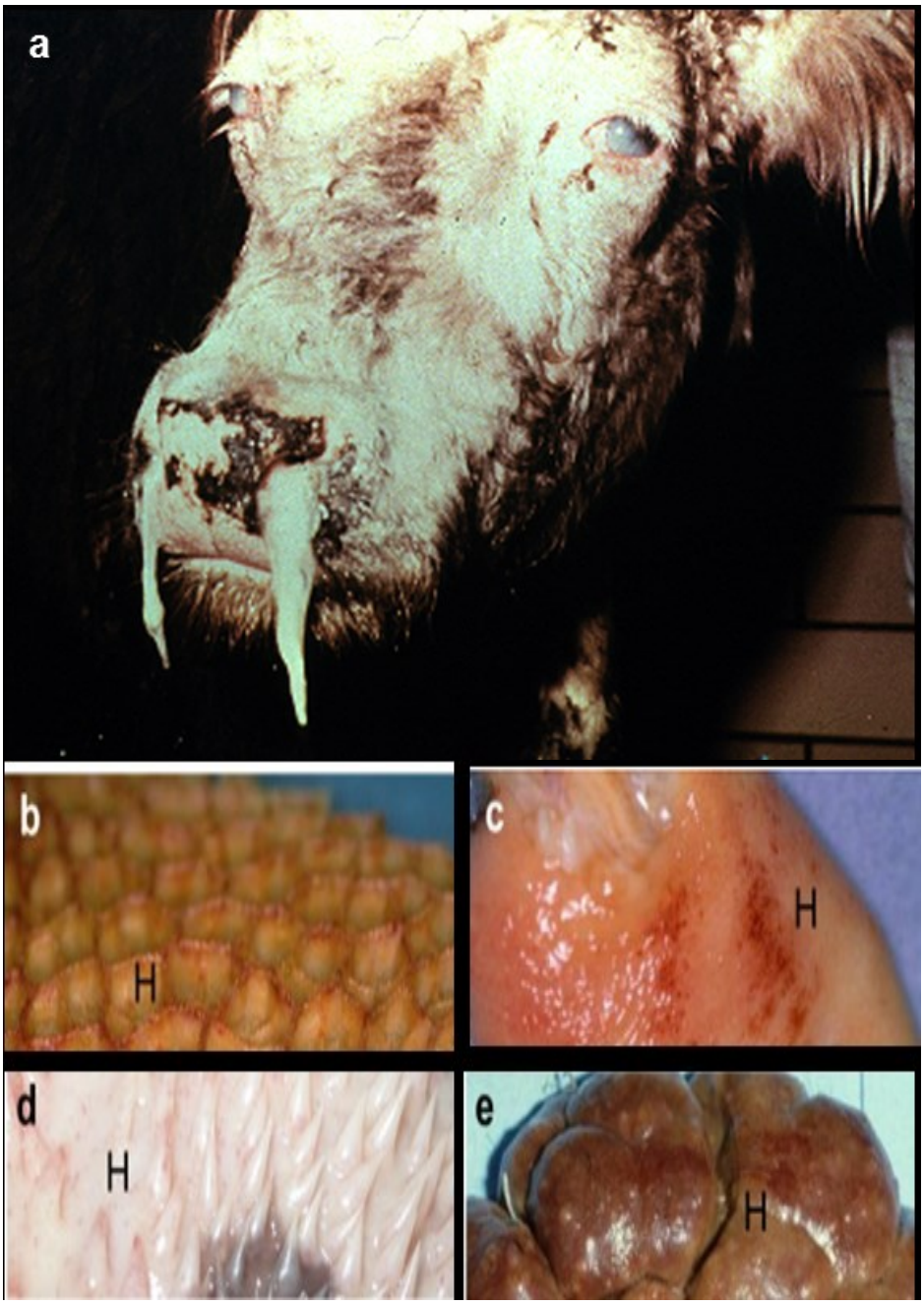


Figure 1.3 Clinical symptoms of MCF

a: An infected cow with a severe head and eye form of MCF. Corneal opacity and nasal discharge can be seen (picture courtesy of Dr Bob Dalziel). b – e: lesions on the reticulum, bladder, buccal papillae and kidney respectively. H indicates areas of haemorrhaging (image adapted from Russell *et al.* 2009).

1.5 Ovine Herpesvirus 2 and Alcelaphine Herpesvirus 1

OvHV-2 and AlHV-1 are the major causative agents of MCF and will be discussed in further detail in the following sections. In the past, the question has been raised whether OvHV-2 and AlHV-1 are in fact the same virus. Development of advanced molecular tools has revealed differences not only at the genome level of these viruses but in their routes of transmission, shedding patterns and pathogenesis leading to the conclusion that OvHV-2 and AlHV-1 are distinct viruses. Unlike AlHV-1, OvHV-2 cannot be propagated *in vitro* and as such studies into OvHV-2 replication and pathogenesis have been highly limited. An experimental infection system of OvHV-2 has been developed using nasal secretions from naturally infected sheep which contain high levels of OvHV-2 (Taus *et al.*, 2006). This model has been used to evaluate OvHV-2 infection in rabbits, bison and sheep (Cunha *et al.*, 2012; Cunha *et al.*, 2013; Li *et al.*, 2008a; Reid *et al.*, 1986).

1.5.1 Genome structure

The genomes of both OvHV-2 and AlHV-1 have been fully sequenced (Ensser *et al.*, 1997; Hart *et al.*, 2007). The unique regions of OvHV-2 and AlHV-1 are both approximately 130 kbp in length. Sequence similarity between OvHV-2 and AlHV-1 ranges from 22% to 83% across the genome. OvHV-2 has 73 predicted ORFs, 62 of which have homology in other γ-herpesviruses. A number of ORFs are unique to OvHV-2 and AlHV-1, which are summarised in Table 1.2. Three ORFs are unique to OvHV-2: Ov2.5 (interleukin-10 homologue), Ov3.5 (unknown function) and Ov8.5 (unknown function). AlHV-1 encodes two unique ORFs A1 and A4 (Hart *et al.*, 2007). Another major difference between the OvHV-2 and AlHV-1 genomes is that OvHV-2 codes for ORF49 whereas AlHV-1 does not. ORF49 has homologues in KSHV EBV, and MHV-68 and is thought to co-operate with ORF50 to induce lytic replication (Lee *et al.*, 2007). A comparison of gammaherpesvirus genomes can be found in Figure 1.4.

Table 1.2 Unique ORFs shared by OvHV-2 and AlHV-1

OvHV-2 ORF	AlHV-1 homologue	Predicted Function
n/a	A1	Unknown function
Ov2	A2	Transcription Factor
Ov2.5	n/a	IL-10 homologue
Ov3	A3	Homologous to proteins of the semaphorin family
Ov3.5	n/a	Unknown function
n/a	A4	Unknown function
Ov4.5	A4.5	B cell lymphoma 2 (Bcl-2) homologue
Ov5	A5	G protein-coupled receptor
Ov6	A6	Transcriptional activator (putative)
Ov7	A7	Glycoprotein
Ov8	A8	Glycoprotein
Ov8.5	n/a	Unknown function
Ov9	A9	Bcl-2 homologue
Ov9.5	A9.5	Secreted glycoprotein
Ov10	A10	Unknown

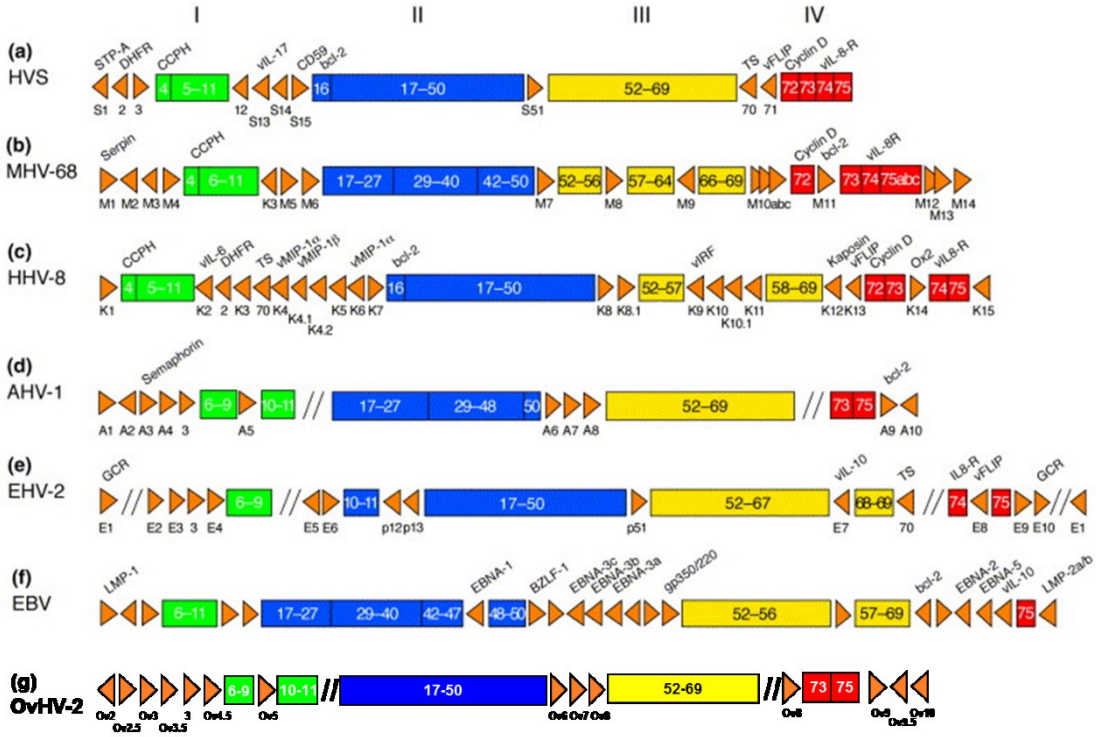


Figure 1.4 A comparison of gammaherpesvirus genomes

Representative genetic maps for seven different gammaherpesviruses. For details of unique genes to alcelaphine herpesvirus 1 (AHV-1) and OvHV-2 see Table 1.2. (Adapted from http://microbewiki.kenyon.edu/images/d/df/Gammaherpesvirus_genomes.jpg.)

1.5.2 Transmission

Transmission of both OvHV-2 and AlHV-1 is thought to occur primarily through inhalation of virus particles, however contact with secretions containing virus particles and contaminated foodstuff and/or water are also possible routes of transmission (Li *et al.*, 2014). Transmission of OvHV-2 from sheep to bison over a distance of 5.1 km has been reported in the literature, emphasising the need for separation of natural and susceptible hosts (Li *et al.*, 2008b). It is unclear whether other factors, such as birds or insects, play a role in this long distance transmission. Both horizontal and vertical transmission have been reported in AlHV-1. Infected wildebeest calves shed virus through nasal secretions. Neutralising antibodies develop around 3 months of age resulting in a decrease in virus shedding. This leads to a seasonal pattern of WA-MCF that occurs during wildebeest calving (Mushi, 1981). The pattern of infection of OvHV-2 differs from AlHV-1 in a number of ways. Firstly, vertical transmission of OvHV-2 is thought to be extremely rare or to not occur at all. Secondly, lambs are generally not infected with OvHV-2 until after two months of age (Li *et al.*, 1998). OvHV-2 can be detected in nasal secretions continuously, however the highest levels of OvHV-2 DNA are found between the ages of six and nine months suggesting that this is the peak time of virus shedding (Li *et al.*, 2001). Thirdly, SA-MCF occurs year-round although a moderate increase in SA-MCF does occur during lambing season. The increase in SA-MCF is not associated with lambing as new-born lambs are not infected with OvHV-2 and there does not appear to be a correlation between parturition and shedding of virus (Harris *et al.*, 1978; Muller-Doblies *et al.*, 2001). A short but intense pattern of shedding is observed for OvHV-2 infected sheep most likely reflecting a single cycle of virus replication (Li *et al.*, 2004). Transmission of AlHV-1 and OvHV-2 does not occur between susceptible species.

1.5.3 OvHV-2 Replication

There are three stages of OvHV-2 replication in sheep: entry, maintenance and exit (Figure 1.4). Primary infection occurs in the lung, specifically alveolar epithelial cells (Taus *et al.*, 2010). ORF25, a late transcript encoding for the major capsid protein, can be detected as early as 1 day post infection (DPI) in lung tissue with the peak of virus DNA and transcripts occurring 7 DPI, and dropping by 9 DPI (Li *et al.*, 2008a). It is thought that this initial replication of OvHV-2 in the lung is required for a switch in cell tropism enabling dissemination and latency; a mechanism similar to that seen in EBV where the ratio of glycoproteins associated with infection of epithelial cells and B cells is altered to allow for more efficient infection of B cells following the initial replication in epithelial cells (Borza & Hutt-Fletcher, 2002). Primary OvHV-2 infection in sheep stimulates a rapid and effective immune response which is thought to clear cells infected with lytic OvHV-2 promoting virus

dissemination in peripheral blood lymphocytes (O'Toole & Li, 2014). During virus dissemination, OvHV-2 DNA is detectable in a wide range of tissues, however ORF25 transcripts are not detectable indicating that OvHV-2 is in a predominantly latent state (Li *et al.*, 2008a). A study using a natural method of OvHV-2 infection of previously uninfected sheep revealed that the primary cells infected during latency are $CD2^+$ lymphocytes. During earlier stages of infection these cells were also $CD4^+$ positive, most likely reflecting a T helper cell population. At later stages of infection, there was a shift towards a $CD8^+$ positive phenotype probably representing a cytotoxic T cell population. OvHV-2 DNA was also detected sporadically in monocytes and other immune cells including B cells and γδ T cells (Meier-Trummer *et al.*, 2010). The trigger for reactivation of latent OvHV-2 is unknown. In sheep actively shedding OvHV-2, ORF25 transcripts can be detected in epithelial cells found in nasal secretions and in turbinate tissues (Cunha *et al.*, 2008; Taus *et al.*, 2010). The unique shedding pattern of OvHV-2 described in section 1.5.2 is in line with another switch in cell tropism which does not allow for reinfection of turbinate epithelial cells.

As there is no transmission between susceptible species there are only two stages of OvHV-2 replication: entry and maintenance (Figure 1.4). Initial infection and replication of OvHV-2 in susceptible species also occurs in the lung. In contrast to infection in sheep, the immune response observed is much smaller with only a subtle increase in immune response transcripts observed in experimentally infected bison compared to sheep (Cunha *et al.*, 2012). Virus dissemination occurs following initial replication, however this is associated with a lytic gene expression pattern with detection of ORF25 and ORF50 transcripts in the majority of tissues in experimentally infected rabbits (Cunha *et al.*, 2013). This has been observed by others who have detected ORF43 and ORF63 (both code for structural proteins) in epithelial cells and M cells of the appendix of experimentally infected rabbits (Meier-Trummer *et al.*, 2009b). The lytic pattern of gene expression in susceptible species indicates a role for virus replication in the development of disease.

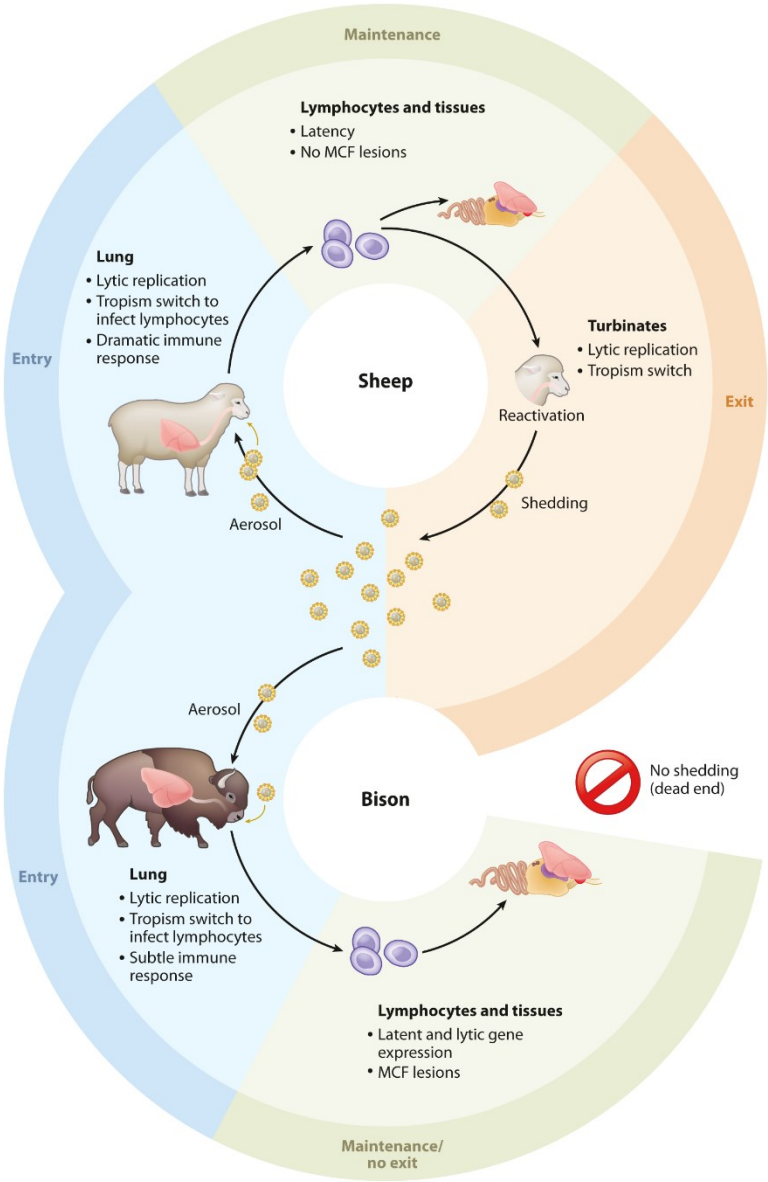


Figure 1.5 OvHV-2 Lifecycles in Sheep and Bison

A cartoon representation of the lifecycle of OvHV-2 in the natural host sheep and susceptible species bison. Three stages (entry, maintenance and exit) exist in sheep however only two of these (entry and maintenance) are seen in susceptible species. Entry in both species is fairly similar however there are large differences in the immune response observed between sheep and bison. Maintenance occurs in the same tissues in both natural and susceptible species however MCF lesions only develop in susceptible species. There is no lytic replication of OvHV-2 observed during maintenance in sheep. Reactivation and subsequent shedding of the virus occurs in sheep. This is not seen in susceptible species which are dead end hosts. Details of the lifecycle of OvHV-2 are found in section 1.5.3. (Republished with permission from Li *et al.* 2014)

1.5.4 Proposed Model of Pathogenesis

The pathology of MCF has been well documented, however what contributes to the development of lesions is not fully understood. Many studies have demonstrated that $CD8^+$ T cells are the predominant cell type associated with lesions leading to the hypothesis that these are the primary cells responsible for the pathology observed in MCF (Dewals & Vanderplasschen, 2011; Ellis *et al.*, 1992; Nakajima *et al.*, 1992; Simon *et al.*, 2003). Lesions from bison experimentally-infected with OvHV-2 were found to contain $CD8^+$, $perforin^+$ γδ T cells (cytotoxic cells of the innate immune system), regulatory $CD4^+$, $perforin^-$ αβ T cells and B cells indicating that pathogenesis of MCF is linked to dysregulation of the immune system (Nelson *et al.*, 2010). OvHV-2 infected cells have a large granular lymphocyte (LGL) phenotype and exhibit non MHC-restricted killing activity and vary in their expression of CD2, CD4 and CD8 (Schock *et al.*, 1998; Swa *et al.*, 2001). LGL cell lines derived from MCF-affected animals, including the cell line BJ1035, were found to be $CD2^+$, $CD3^+$ and $CD6^+$ (general T cell markers) with a proportion of cells positive for the scavenger receptor workshop cluster 1 (WC1). They were $CD4^-$, $CD8^-$ and were negative for γδ T cell, B cell and natural killer cell markers (Dr Inga Dry, personal communication). BJ1035 cells are thought to constitutively express tumour necrosis factor α (TNFα), interferon γ (IFNγ), IL-10 and IL-4 however no expression of IL-1β or IL-2 has been detected consistent with the hypothesis that OvHV-2 infected cells have an unregulated cytotoxic cell phenotype (Schock *et al.*, 1998; Swa *et al.*, 2001).

IL-2 is known as a major T cell growth factor and it is required for the *in vitro* growth of T cell lines, including the BJ1035 cell line mentioned previously (Kundig *et al.*, 1993). However, some studies have challenged this view as mice lacking IL-2 or receptor genes are not immunocompromised and instead develop an autoimmune disease (Almeida *et al.*, 2002; Kramer *et al.*, 1995). A number of hypotheses for this contradiction have been postulated; the major two of which are the role of IL-2 in promotion of activation-induced cell death (AICD) and the requirement of IL-2 for the establishment of stable and sizeable populations of regulatory T cells (Almeida *et al.*, 2002; Lenardo, 1991). Although IL-2 is able to promote AICD *in vitro* it has been shown that this may not be the major mechanism *in vivo* and that the major role of IL-2 *in vivo* is to promote thymic development and peripheral expansion of regulatory T cells (Nelson, 2004). The clinical signs observed in MCF are consistent with the phenotype of IL-2 deficient mice described. Significantly lower levels of IL-2 have been detected in tissues of SA-MCF affected animals compared to their healthy counterparts (Cunha *et al.*, 2012; Cunha *et al.*, 2013; Meier-Trummer *et al.*, 2009a). In cattle experimentally infected with OvHV-2, IL-2 transcript was far less abundant than in healthy animals and it was

hypothesised that down-regulation of IL-2 transcription may lead to an IL-2 deficiency and may be an important factor in pathogenesis of MCF (Meier-Trummer *et al.*, 2009a).

Although many studies have investigated the populations of immune cells associated with lesions, little research has been done into the role the virus plays in MCF pathogenesis. One study has demonstrated the presence of OvHV-2 infected $CD8^+$ lymphocytes in lesions from SA-MCF affected cattle and bison (Simon *et al.*, 2003). ORF25 protein has been found in fibroblasts in vascular lesions from bison with SA-MCF (Nelson *et al.*, 2013). Moreover, the severity of MCF lesions positively correlates with ORF25 expression levels in tissues of SA-MCF affected bison and rabbits (Cunha *et al.*, 2012; Cunha *et al.*, 2013). It is therefore likely that the virus has a direct role in pathogenesis, as well as indirect dysregulation of the immune system leading to the development of lesions. It is important to note that there have been reports of subclinical infection in the absence of clinical disease in both cattle and bison (O'Toole *et al.*, 2002; Powers *et al.*, 2005). This complicates matters even further, as there may be other extraneous factors involved in the pathogenesis of MCF including genetic susceptibility (Traul *et al.*, 2007). Experimentally, it has been shown that different titres of OvHV-2 inoculum can result in different outcomes of infection, from a negative result through subclinical infection and development of MCF (Li *et al.*, 2014)

A number of differences in pathogenesis have been observed between OvHV-2 and AlHV-1. In SA-MCF affected animals, proliferation of $CD8^+$ T cells, $CD4^+$ T cells and B cells in peripheral blood has been observed whereas in WA-MCF affected animals, proliferation of only $CD8^+$ T cells in peripheral blood has been observed (Dewals *et al.*, 2008; Li *et al.*, 2011a). The development of MCF in AlHV-1 infected animals is predominantly associated with latent infection. In rabbits experimentally infected with AlHV-1, ORF73 was readily detected in infected tissue (Dewals *et al.*, 2008). Furthermore, a role for ORF73 in the pathogenesis of MCF has been described. A mutated AlHV-1 lacking ORF73 was produced which did not impair initial virus replication but abrogated pathogenesis and development of MCF in experimentally-infected calves (Palmeira *et al.*, 2013). This latency-associated pathogenesis of WA-MCF is in contrast to SA-MCF in which tissues from experimentally-infected rabbits contained the lytic transcripts ORF25 and ORF50 (Li *et al.*, 2011a). However, conflicting data from euthanised cattle infected with OvHV-2, suggested a latent profile of infection as only ORF73 could be detected in affected tissues (Meier-Trummer *et al.*, 2009a). The differences observed between these two studies could be due to differences in disease stage, the sensitivity of the detection methods used or individual susceptibility and further research into this is clearly warranted.

1.6 microRNAs

miRNAs are short (~22 nt) non-coding RNAs that regulate gene expression at the post transcriptional level (Bartel, 2004). The first miRNA was discovered simultaneously by two groups researching the development of *Caenorhabditis elegans* (*C. elegans*). It was shown that the *lin-4* gene did not code for protein but produced a small RNA that was antisense to the 3' UTR of the *lin-14* gene resulting in repression of this gene (Lee *et al.*, 1993; Wightman *et al.*, 1993). However the field of miRNA research did not really begin until a second miRNA, *let-7*, was discovered, also in *C. elegans* in 2000 (Reinhart *et al.*, 2000). The discovery of these two small RNAs led to the naming of miRNAs and research began to investigate whether other species had miRNAs and what roles they played in biology (Lee & Ambros, 2001). To date there are 35828 mature miRNAs in 223 species within the miRNA database miRbase (http://www.mirbase.org/). These species include algae, protozoa, arthropods, nematodes, mammals, plants and viruses (Bartel, 2004; Grundhoff & Sullivan, 2011; Lee *et al.*, 2004b). miRNAs have been shown to function in nearly every biological process ranging from development, cell fate determination, cell proliferation and death to the immune response and disease development (Bhatt *et al.*, 2011; Dong *et al.*, 2013; Stark *et al.*, 2003). In fact, over 60% of the human protein-coding genes have been shown to contain at least one conserved miRNA binding site (Ha & Kim, 2014).

1.6.1 miRNA biogenesis

The biogenesis and processing of miRNAs is a complex multi-step process (Figure 1.5). The majority of miRNA genes are transcribed by RNA polymerase II (pol II), although some are transcribed by RNA polymerase III (Lee *et al.*, 2002; Monteys *et al.*, 2010). These primary transcripts are typically over 1 kb in length, have a 5' cap and 3' polyadenylation and are termed primary miRNAs (pri-miRNAs) (Cai *et al.*, 2004; Ha & Kim, 2014). The majority of pri-miRNAs are located in intergenic regions of the genome or in an antisense direction to annotated genes, however some are located in introns and exons (Ha & Kim, 2014; Kim, 2005; Lee *et al.*, 2004a). pri-miRNAs are capable of generating a single miRNA (monocistronic) or a number of miRNAs (polycistronic transcript); these can be under a single promoter or multiple promoters for each individual miRNA (Cai *et al.*, 2004; Lee *et al.*, 2002; Song & Wang, 2008). Structurally, pri-miRNAs include an imperfect dsRNA stem loop flanked by single stranded RNA. One arm of the stem loop structure contains the mature miRNA sequence (Zeng & Cullen, 2005).

Processing of pri-miRNAs occurs through interactions with the microprocessor complex. The microprocessor complex consists of the RNase III enzyme Drosha and its cofactor protein

microprocessor complex subunit DGCR8 (encoded by DiGeorge syndrome critical region gene 8) (Beezhold *et al.*, 2010; Davis-Dusenbery & Hata, 2010; Landthaler *et al.*, 2004). DGCR8 interacts with the stem loop structure of the pri-miRNA and recruits Drosha, which results in the cleavage of the pri-miRNA at the stem loop structure liberating a 60 – 100 nucleotide (nt) dsRNA loop structure termed a precursor-miRNA (pre-miRNA). Important structural features of pre-miRNAs include a 5' phosphate and a 2 nt overhang at the 3' end (Kim *et al.*, 2009; Zeng & Cullen, 2005; Zeng *et al.*, 2005). The 3' overhang feature is recognised by the Exportin/Ran complex (consisting of Exportin 5 and Ran-GTP co-factor) facilitating export of the pre-miRNA from the nucleus into the cytoplasm (Bohnsack *et al.*, 2004).

Hydrolysis of Ran-GTP to Ran-GDP releases the pre-miRNA from the export complex (Wang *et al.*, 2011). The pre-miRNA then interacts with the RNA induced silencing complex (RISC) loading complex (RLC). The RLC is comprised of the RNase III enzyme Dicer and its cofactors TAR RNA‐binding protein (TRBP), protein activator of PKR (PACT) and the argonaute-2 (Ago-2) protein (Gregory *et al.*, 2005; Haase *et al.*, 2005; Lee *et al.*, 2006; MacRae *et al.*, 2008). Although not absolutely necessary for pre-miRNA processing, TRBP and PACT play roles in stabilisation of Dicer, Ago-2 recruitment and formation of the RLC (Chendrimada *et al.*, 2005; Kok *et al.*, 2007; Lee *et al.*, 2006). The pre-miRNA is recognised by the piwi-argonaute-zwille (PAZ) and RNase IIIa and RNase IIIb domains of Dicer. Cleavage of the pre-miRNA stem loop structure is carried out by Dicer resulting in an approximately 22 nt dsRNA duplex with a 5' phosphate and a 3' hydroxyl group with 2 nt overhangs at the 3' ends (Lingel *et al.*, 2003; MacRae *et al.*, 2007; Yan *et al.*, 2003; Zhang *et al.*, 2004).

The effector complex RISC is generated through interaction of the miRNA duplex with an Ago protein (Gregory *et al.*, 2005; Hutvágner & Zamore, 2002; Mourelatos *et al.*, 2002). The Ago proteins are the key effector molecules of the RISC and contain PAZ, middle (MID) and P-element-induced whimpy testes (PIWI) domains (Maniataki *et al.*, 2005). The PAZ domain recognises the 2 nt overhang at the 3' ends and the MID domain acts to anchor the 5' ends of the miRNA duplex (Jinek & Doudna, 2009; Lingel *et al.*, 2004; Ma *et al.*, 2004). The PIWI domain has a similar structure to RNase H and is thought to play a role in the cleavage of the target mRNA (Kim *et al.*, 2009; Ma *et al.*, 2005; Parker *et al.*, 2005). There are four Ago proteins in mammals (Ago-1 to -4) however only Ago-2 has been found to have an enzymatically active PIWI domain capable of cleaving target mRNAs with perfect complementarity to the mature miRNA (Liu *et al.*, 2004; Meister *et al.*, 2004).

Upon loading of the miRNA duplex into the RISC, the duplex is unwound by helicases. Only one strand remains in the RISC and becomes the mature miRNA or guide strand. The other strand (or passenger strand) is released from the RISC and is either degraded or incorporated into other RISCs where it can act as another mature miRNA (Ghildiyal *et al.*, 2010; Hutvágner & Zamore, 2002; Kim *et al.*, 2009; Okamura *et al.*, 2008). The thermodynamic stability of the strands of the duplex dictate which strand is selected as the guide strand. The strand with less stable base pairing at the 5' end becomes the mature miRNA (Khvorova *et al.*, 2003). If both strands are capable of acting as mature miRNAs then 5p or 3p is added to the name of the miRNA to denote from which strand of the duplex the miRNA originated (Griffiths-Jones *et al.*, 2006).

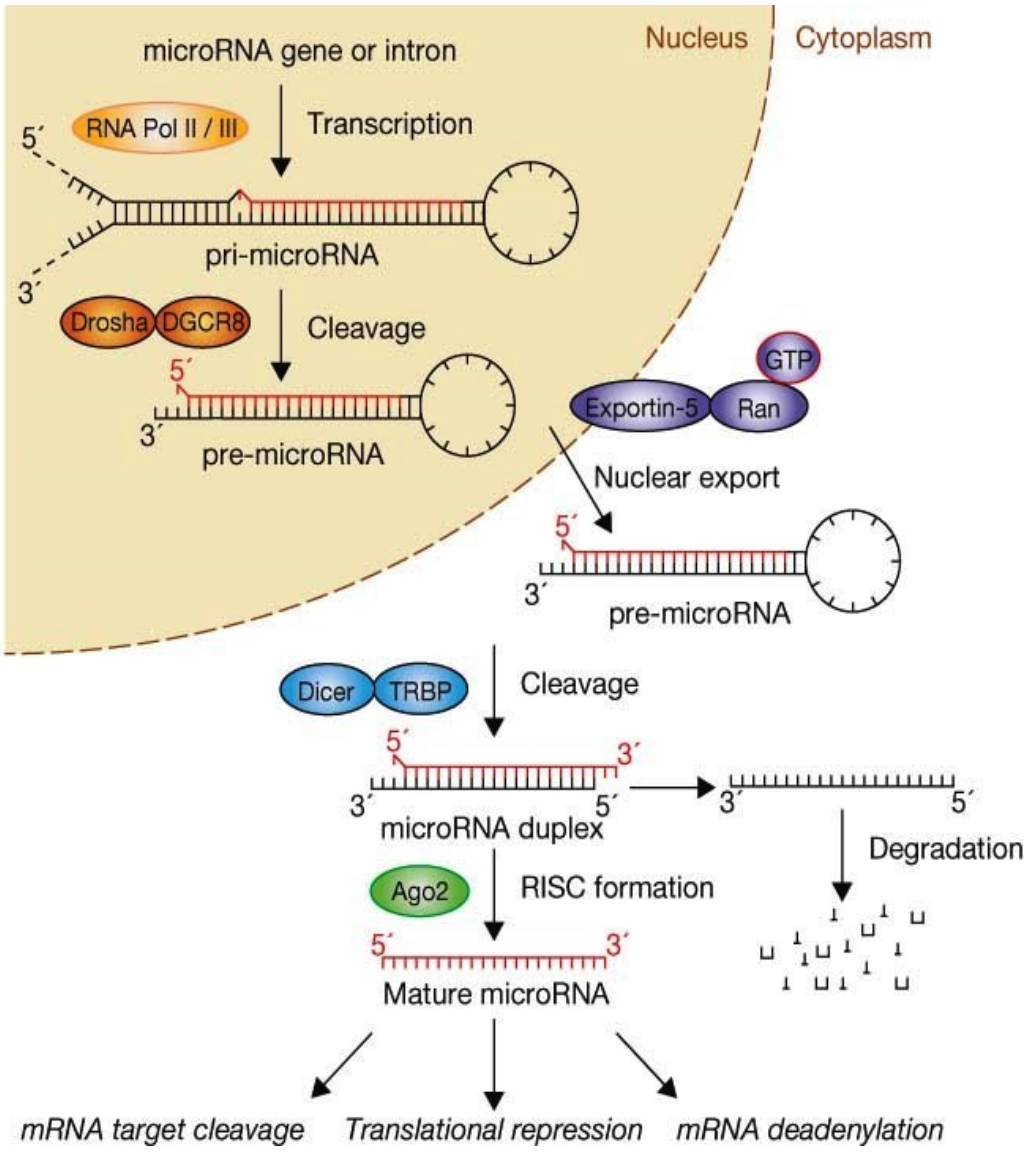


Figure 1.6 miRNA biogenesis pathway

See section 1.6.1 for details of the miRNA biogenesis pathway. Adapted from Winter *et al*., 2009.

1.6.2 Target Recognition of miRNAs

miRNA-mediated gene regulation occurs through binding of the miRNA to a target mRNA in a sequence specific manner. The level of complementarity between the miRNA and target mRNA can vary and this dictates the outcome of miRNA binding. For example, miRNAs encoded by plants often show perfect or near perfect complementarity to their target mRNAs resulting in translational repression through degradation of the target mRNA (Brodersen *et al.*, 2008). In animals (and viruses), it is thought that a six to eight nt sequence (termed the seed sequence) located at the 5' end of the miRNA (usually nucleotides 2-8) is most important in target mRNA recognition (Gottwein & Cullen, 2007). Binding of the miRNA to a target mRNA through the seed sequence is known as canonical binding and it is this seed sequence which is most conserved across different species (Lewis *et al.*, 2005; Lim *et al.*, 2005). Although the majority of target recognition by miRNAs involves binding at the 5' end, non-canonical interactions can occur. Examples of this include bulges in the seed sequence, binding of only the 3' end or centre of the miRNA and mismatched nucleotides (Helwak *et al.*, 2013; Lee *et al.*, 2009; Shin *et al.*, 2010). Helwak *et al.* used a technique called Cross-linking Ligation and Sequencing of Hybrids (CLASH) to elucidate targets of human miRNAs and discovered that in fact 60% of the interactions between miRNAs and mRNAs were non-canonical. Furthermore, 18% of interactions involved only base pairing at the 3' end with no 5' base pairing involved (Helwak *et al.*, 2013). Mismatched nucleotide base pairing, usually G:U pairing (instead of the canonical G:C pairing), can occur in the seed sequence of miRNAs and result in functional activity, however, it is thought that this does affect the level of target repression (Brennecke *et al.*, 2005; Didiano & Hobert, 2006; Doench & Sharp, 2004). It was originally thought that the action of miRNAs was through binding to the 3'UTR of a target mRNA however other groups have since shown that binding in the 5'UTR and coding region is possible (Grey *et al.*, 2010; Lee *et al.*, 2009; Lytle *et al.*, 2007; Riaz *et al.*, 2014).

1.6.3 Modes of Action of miRNAs

The major two modes of action of miRNAs are translational repression and degradation of target mRNAs. miRNA-mediated translational repression can occur at the translation initiation or post-initiation stages (Chekulaeva & Filipowicz, 2009; Filipowicz *et al.*, 2008; Huntzinger & Izaurralde, 2011; Hussain, 2012). It has been demonstrated that mRNAs that do not have a 5' cap show some resistance to miRNA-mediated translational repression indicating that silencing interferes with the cap recognition process or binding of the eukaryotic translation initiation factor 4E (eIF4E) to the cap (Humphreys *et al.*, 2005; Mathonnet *et al.*, 2007; Meijer *et al.*, 2013; Pillai *et al.*, 2005; Wakiyama *et al.*, 2007). Furthermore, it has been postulated that Ago-2 interacts with the 5' cap of the target mRNA preventing the binding of eIF4E and

thus initiation of translation (Mathonnet *et al.*, 2007). Many studies have demonstrated the presence of repressed mRNAs associated with actively translating polysomes or in polysomal fractions suggesting that repression of translation can occur post-initiation, either by degradation of the nascent polypeptide chain or by initiating premature ribosome drop off from the target mRNA (Maroney *et al.*, 2006; Nottrott *et al.*, 2006; Olsen & Ambros, 1999; Petersen *et al.*, 2006).

Translational repression can be associated with destabilisation and subsequent degradation of the target mRNA. This process usually requires deadenylation and/or 5' decapping (Behm-Ansmant *et al.*, 2006; Eulalio *et al.*, 2009; Humphreys *et al.*, 2005). Degradation of the target mRNA is thought to occur in P-bodies (Eulalio *et al.*, 2007; Liu *et al.*, 2005; Parker & Sheth, 2007). Proteins involved in the deadenylation and degradation process have been well characterised, however the exact mechanism of recruitment of these proteins to the RISC has not been elucidated (Braun *et al.*, 2011; Fabian *et al.*, 2011; Fabian *et al.*, 2009; Hussain, 2012; Yamashita *et al.*, 2005; Zekri *et al.*, 2009). Decapping of target mRNAs occurs through the canonical mechanism by recruitment of the decapping complex and subsequent exonuclease activity to degrade the mRNA (Cougot *et al.*, 2004; Rehwinkel *et al.*, 2005). miRNA-mediated translational activation can be achieved in two ways; direct activation of the target mRNA by the miRNA or through abrogation of a repressive miRNA leading to relief of repression (Lin *et al.*, 2011; Vasudevan, 2012; Vasudevan *et al.*, 2007).

1.6.4 Approaches for miRNA target identification

Since their discovery, a major area of research has been target identification and the functional analysis of miRNAs. Elucidation of targets of miRNAs and understanding their role in biology has proved to be challenging. Several approaches to identify targets of miRNAs have been applied including both bioinformatic and experimental approaches however a combination of these appears to be the most successful.

A number of computer programmes have been developed to aid miRNA target identification (Peterson *et al.*, 2014). These programmes include miRanda, (John *et al.*, 2004) PicTar (Lall *et al.*, 2006), TargetScan (Ruby *et al.*, 2007), PITA (Kertesz *et al.*, 2007), DIANA-microT (Kiriakidou *et al.*, 2004), rna22 (Miranda *et al.*, 2006) and RNAHybrid (Rehmsmeier *et al.*, 2004). All of these algorithms use slightly different parameters to identify targets of miRNAs including the sequence complementarity of the seed sequence of the miRNA and target mRNA, thermodynamic stability and conservation of the target site between orthologous genes. As such, it is often difficult to identify the same target of a certain miRNA in different programmes. Also, some programmes are only capable of predicting targets of miRNAs in the

3'UTRs of mRNAs. Another limitation of using a bioinformatic approach is that a significant number of false positives are generated and so experimental validation of targets is required.

There are three main experimental approaches to miRNA target identification: transcriptome analysis, biochemical approaches and proteomic analysis. Transcriptome analysis allows for the measurement of gene expression in the presence or absence of a specific miRNA. The most common form of transcriptome analysis is a microarray, however RNA-seq can also be used (Donohoe *et al.*, 2015; Jia *et al.*, 2015; Kanda *et al.*, 2015; Ma *et al.*, 2015; Ramalingam *et al.*, 2015; Xing *et al.*, 2015). The limitations of using a microarray or RNA-seq include not being able to directly identify the target mRNA(s) of the miRNA(s) of interest as only overall changes in gene expression are determined.

Using a biochemical approach can attempt to overcome the limitation of transcriptome analysis as these methods use the principle of purifying miRNAs bound to their target mRNAs through immunoprecipitation (IP) of an Ago protein in the RISC (RISC-IP) (Beitzinger *et al.*, 2007; Grey *et al.*, 2010). The immunoprecipitated mRNAs can then be identified by microarray or RNA-seq approaches. Limitations of this method include indirect mRNA targets of miRNAs pulled down due to the low stringency of the IP method and specific interacting sites of miRNAs are not identified.

The RISC-IP method has been improved upon with the development of the high throughput sequencing of RNAs isolated by UV crosslinking and immunoprecipitation (HITS-CLIP) method (Chi *et al.*, 2009; Moore *et al.*, 2014). By crosslinking the miRNA-mRNA complex to Ago-2 a higher stringency can be used in this technique to increase confidence in the mRNA targets identified. A number of targets of KSHV-encoded and EBV-encoded miRNAs have been identified using this method (Haecker *et al.*, 2012; Haecker & Renne, 2014; Riley *et al.*, 2012). HITS-CLIP has been improved through the development of the photoactivable-ribonucleoside-enhanced-CLIP (PAR-CLIP) method. The PAR-CLIP method has an improved efficiency of crosslinking increasing the amount of mRNA pulled down compared to HITS-CLIP (Hafner *et al.*, 2010). A number of groups have used PAR-CLIP to identify targets of KSHV-encoded and EBV-encoded miRNAs (Gottwein *et al.*, 2011; Skalsky *et al.*, 2012). However, neither HITS-CLIP nor PAR-CLIP are capable of definitively identifying targets of miRNAs.

Most recently, CLASH has been developed as a method of miRNA target identification that overcomes the limitations of previous CLIP methods. An intramolecular ligation step is included in the CLASH protocol to ligate miRNAs to their target mRNAs resulting in miRNA-mRNA hybrids that can be identified by subsequent sequencing (Helwak *et al.*, 2013). CLASH

has mainly been used to map the human miRNA interactome however CLASH has been used to identify targets of OvHV-2-encoded miRNAs by the Dalziel lab (see section 4.1) (Friedländer *et al.*, 2014; Helwak *et al.*, 2013; Helwak & Tollervey, 2014; Liu *et al.*, 2014; Riaz, 2014).

Proteomic approaches to miRNA target identification include measuring the levels of newly synthesised proteins using (pSILAC) (Ebner & Selbach, 2011; Kaller *et al.*, 2014). This method is of particular use when the decrease in protein from miRNA-mediated repression is modest and would not be detected by other proteomic or transcriptomic approaches.

1.7 Virus-encoded miRNAs

It is well known that viruses modulate a number of host pathways to create a cellular environment that favours the virus for facilitation of replication and evasion of the immune response. It is therefore not surprising that it was hypothesised that viruses might encode miRNAs to help mediate these subversive processes (Sullivan, 2008; Tuddenham & Pfeffer, 2006). Moreover, miRNAs are an ideal mechanism for viruses to modulate the host as they are non-immunogenic and occupy little genome space (Sarnow *et al.*, 2006).

The first virus-encoded miRNAs were identified in EBV and it was shown that the encoded miRNAs were differentially expressed depending on the life cycle stage of the virus (Pfeffer *et al.*, 2004). The current release of miRbase lists 498 mature miRNAs from 27 different viruses (Table 1.3). The majority of viruses that encode miRNAs are DNA viruses. Most DNA viruses replicate in the nucleus and so have access to the necessary machinery for correct miRNA processing; DNA viruses that replicate in the cytoplasm such as poxviruses might not have evolved to code for miRNAs due to their inability to access the processing enzyme Drosha (Cullen, 2010; Kincaid & Sullivan, 2012; Tuddenham & Pfeffer, 2006). DNA viruses also often have larger genomes than RNA viruses and so have the coding capacity for miRNAs. There is very little evidence that RNA viruses encode miRNAs, although it has been shown that Dengue virus and West Nile virus encode miRNA-like molecules (Hussain & Asgari, 2014; Hussain *et al.*, 2012; Ospina-Bedoya *et al.*, 2014). There is also controversial evidence that HIV-1 encodes a number of miRNAs (Bennasser *et al.*, 2004; Omoto *et al.*, 2004) however some studies have contradicted these findings (Lin & Cullen, 2007; Pfeffer *et al.*, 2005). However another retrovirus, bovine leukaemia virus (BLV), clearly encodes a number of miRNAs (Rosewick *et al.*, 2013). These miRNAs are clustered in the BLV genome and are transcribed by RNA polymerase III. (Kincaid *et al.*, 2012).

The majority of virus-encoded miRNAs (over 90%) discovered so far are encoded by herpesviruses (Grundhoff & Sullivan, 2011). The functional targets of a large percentage of herpesvirus-encoded miRNAs remain unknown, however targets that have been elucidated include both virus and cellular mRNAs and play roles in latency, immune evasion and pathogenesis. Tables 1.4 and 1.5 show selected known cellular targets and viral targets of herpesvirus-encoded miRNAs respectively. It is out with the scope of this thesis to discuss all the known targets and so a select number will be discussed in the following sections.

Table 1.3 Virus-encoded miRNAs

Family	Subfamily	Virus	No. mature miRNAs
Herpesvirus	α-herpesvirus	bovine herpesvirus 1	12
		bovine herpesvirus 5	5
		fuck enteritis virus	33
		herpes B virus	15
		herpesvirus of turkeys	28
		HSV-1	27
		HSV-2	24
		ILTV	10
		MDV-1	26
		MDV-2	36
		pseudorabies virus	13
	β-herpesvirus	HCMV	26
		HHV-6B	8
		MCMV	29
	γ-herpesvirus	EBV	44
		HVS	6
		KSHV	25
		MHV-68	28
		rhesus lymphocryptovirus	68
		rhesus monkey rhadinovirus	11
Papillomavirus		BPCV1	1
		BPCV2	1
Polyomavirus		BK polyomavirus	2
		JC polyomavirus	2
		Merkel cell polyomavirus	2
		simian virus 40	2
Retrovirus		BLV	10
		bovine foamy virus	4

Adapted from miRbase (v21 June 2014) (http://www.mirbase.org/)

1.7.1 Cellular targets of Herpesvirus-encoded miRNAs

Many of the known cellular targets of herpesvirus-encoded miRNAs are involved in the maintenance of latency through a number of pathways such as prevention of apoptosis and immune evasion (Table 1.4). Regulation of the cell cycle can also be important, not only for maintenance of latency but also for pathogenesis and oncogenesis in herpesviruses such as KSHV.

A number of herpesviruses encode one or more miRNAs known to target the apoptosis pathway. The EBV-encoded miRNA miR-BART5 has been shown to target p53-up-regulated modulator of apoptosis (*PUMA*), the protein product of which belongs to the B cell lymphoma 2 (Bcl-2) family of proteins (Choy *et al.*, 2008). HCMV and KSHV encode miRNAs (HCMV-miR-UL112-1 and KSHV-miR-K5, -miR-K9 and -miR-K10a/b) that target the Bcl-2 associated transcription factor 1 (*BCLAF1*), the protein of which is another promotor of apoptosis (Sarras *et al.*, 2010; Ziegelbauer *et al.*, 2009). KSHV also targets the apoptosis pathway through targeting of a number of other proteins. KSHV-miR-K12-1, miR-K12-4-3p and miR-K12-3 have all been shown to target caspase 3 (*CASP3*), the protein product of which is involved in the activation of the cascade of caspases that trigger apoptosis (Suffert *et al.*, 2011). KSHV-miR-K12-1 has also been shown to target *MAF* (V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog), which codes for a transcription factor which has several roles including increasing the susceptibility of T cells to apoptosis. KSHV-miR-K12-6-5p and miR-K12-11 (a miR-155 orthologue, see section 4.1 for further details) have also been shown to target *MAF* (Hansen *et al.*, 2010). It has been demonstrated that KSHV-miR-K12-10a can down-regulate tumour necrosis factor receptor superfamily member 12A (tweak-receptor or *TWEAKR*), which codes for a receptor for the cytokine tumour necrosis factor ligand superfamily member 12 (TNFSF12). TNFSF12 has some overlapping features with the TNF signalling pathway and, as the name suggests, induces weak apoptosis in certain cell types (Abend *et al.*, 2010). *SMAD5*, the protein product of which is a component of the TGFβ signalling pathway has shown to be a target of KSHV-miR-K12-11 (Liu *et al.*, 2012). TGFβ is a potent inhibitor of cell proliferation as well as a regulator of many other cell processes such as differentiation and apoptosis. Another SMAD gene, *SMAD2*, has been shown to be targeted by an MDV-encoded miRNA MDV-miR-3; down-regulation of *SMAD2* has been reported to suppress cisplatin-induced apoptosis (Xu *et al.*, 2011).

Immune evasion is thought to be critical for maintenance of latency and so it is not surprising that a number of herpesviruses encode multiple miRNAs that suppress or subvert the immune system. HCMV, EBV and KSHV all encode miRNAs (HCMV-miR-UL112-1, KSHV-miR-

K-12-7 and EBV-miR-BART2-5p) that target MHC Class I Polypeptide-Related Sequence B (*MICB*) (Nachmani *et al.*, 2009; Stern-Ginossar *et al.*, 2012). MICB is a ligand for the activating natural killer (NK) cell receptor natural-killer group 2, member D (NKG2D). Down-regulation of MICB leads to an inhibition of NK cells and thus reduced recognition and killing of virus infected cells. HCMV also codes for miR-UL148D, which targets *RANTES* (regulated on activation, normal T cell expressed and secreted), which codes for a chemokine which acts as a chemoattractant for blood monocytes, memory T helper cells and eosinophils (Kim *et al.*, 2012). It is a known major suppressor of HIV replication (Coffey *et al.*, 1997). Another chemokine gene, chemokine (C-X-C motif) ligand 11 (*CXCL11*), is targeted by EBV-miR-BHRF1-3 (Xia 2008). Gene expression of *CXCL11* is potently induced by IFNγ and as such is thought to have antiviral effects (Murayama *et al.*, 2012). KSHV-miR-K-12-9 targets *IRAK1* (interleukin-1 receptor-associated kinase 1) and *MYD88* (Myeloid differentiation primary response gene 88). The protein products of both of these genes are involved in the IL-1 and Toll-like Receptor signalling pathways. IRAK1 associates with Myd88 and downstream signalling leads to NF-κB activation and the transcription of type I IFNs thus playing a critical role in the initiation of the innate immune response (Abend *et al.*, 2012).

Other notable targets of herpesvirus-encoded miRNAs include those that target cell cycle genes. *CCNE2* (cyclin E2), which codes for a protein that plays a role in the G_1/S phase transition is targeted by HCMV-miR-US25-1 (Grey *et al.*, 2010). KSHV encodes two miRNAs, miR-K12-1 and miR-K12-4-5p, which target *P21* (codes for a regulator of G_1 progression) and *RBL2* (Retinoblastoma-Like 2, the protein product of which is a regulator of entry into cell division) respectively (Gottwein & Cullen, 2010; Lu *et al.*, 2010a). Four KSHV-encoded miRNAs, miR-K12-1, miR-K3-3p, miR-K6-3p and miR-K11 target thrombospondin 1 (*THBS1*) (Lu *et al.*, 2010a; Samols *et al.*, 2007). THBS1 is an adhesive glycoprotein that mediates cell-cell and cell-matric interactions and has been shown to play roles in angiogenesis and tumourigenesis.

Table 1.4 Cellular targets of herpesvirus-encoded miRNAs

miRNA Target	Virus	miRNA	miRNA Target	Virus	miRNA
BACH1	KSHV	miR-K12-11	MyD88	KSHV	miR-K12-9
BCLAF1	KSHV	miR-K5	NFIB	KSHV	miR-K12-3
		miR-K9		KSHV	miR-K12-7
		miR-K10a/b		KSHV	miR-K12-11
	HCMV	miR-UL112-1	p21	KSHV	miR-K12-1
CASP3	KSHV	miR-K12-1	PU.1	MDV1	miR-M4
	KSHV	miR-K12-4-3p	PUMA	EBV	miR-BART5
	KSHV	miR-K12-3	RANTES	HCMV	miR-UL148D
CCNE2	HCMV	miR-US25-1	Rbl2	KSHV	miR-K12-4-5p
CXCL-11	EBV	mir-BHRF1-3	SMAD2	MDV1	miR-M3
GPM6B	MDV1	miR-M4	SMAD5	KSHV	miR-K12-11
IRAK1	KSHV	miR-K12-9	THBS1	KSHV	miR-K12-1
MAF	KSHV	miR-K12-1			miR-K3-3p
		miR-K12-6-5p			miR-K6-3p
		miR-K12-11			miR-K11
MAP3K2	EBV	miR-BART-18-5p	TWEAKR	KSHV	miR-K12-10a
MICB	HCMV	miR-UL112-1			
	KSHV	miR-K12-7			
	EBV	miR-BART2-5p			

Targets in red are genes associated with apoptosis. Targets in purple are associated with the cell cycle. Targtes in blue are associated with the immune system. Targets in black include genes involved in other signalling pathways and tumourigenesis. Adapted from Grey 2015 and Grundhoff *et al.*, 2011.

1.7.2 Viral targets of Herpesvirus-encoded miRNAs

As well as regulating a number of cellular genes, herpesviruses have been shown to regulate their own gene expression through miRNAs (Table 1.5). Many herpesviruses encode miRNAs that target IE genes. As the majority of herpesvirus-encoded miRNAs are thought to be expressed during latency it is thought that this targeting is a mechanism of maintenance of latency however down-regulation of IE genes could also be a mechanism of promoting or establishing latency. The major transactivators of KSHV (RTA), EBV (BZLF1 and BRLF1), HCMV (IE72/IE1) and MDV-1 (ICP4) are targeted by KSHV-miR-K12-9* and miR-K12-7-5p, EBV-miR-BART20-5p, HCMV-miR-UL112-1 and MDV-miR-M7-5p respectively (Bellare & Ganem, 2009; Grey *et al.*, 2007; Jung *et al.*, 2014; Lei *et al.*, 2010; Lu *et al.*, 2010a; Murphy *et al.*, 2008; Strassheim *et al.*, 2012). The DNA polymerase of EBV, BALF5, is also targeted by an EBV-encoded miRNA, miR-BART2 (Barth *et al.*, 2008). ICP27, the MDV-1 homologue of ORF57 is also targeted by MDV-miR-M7-5p (Strassheim *et al.*, 2012). MDV-miR-M4 targets UL28 and UL32, involved in cleavage and packaging of viral DNA (Muylkens *et al.*, 2010). EBV encodes miRNAs that target genes involved in latency; LMP1 is a target of EBV-miR-BART1-5p, miR-BART16 and miR-BART17-5p. The expression of LMP1 is thought to be under tight regulation because it has been shown that overexpression of LMP1 can lead to growth inhibition and apoptosis which would obviously not be favourable for EBV (Lo *et al.*, 2007). LMP2A, another latency associated protein, is targeted by EBV-miR-BART22. LMP2A is a strongly immunogenic antigen, and that down-regulation of LMP2A by miR-BART22 may lead to the escape of EBV from the immune system and may facilitate NPC progression (Lung *et al.*, 2009).

Taken together it appears that herpesvirus-encoded miRNAs play critical role in regulation of both cellular and viral gene expression to module a number of pathways facilitating pathogenesis of these viruses.

Table 1.5 Viral targets of herpesvirus-encoded miRNAs

Virus	miRNA	Target Gene	Function
EBV	miR-BART22	LMP-2a	Latency
EBV	miR-BART1-5p	LMP1	Latency
	miR-BART16		
	miR-BART17-5p		
EBV	miR-BART2	BALF5	DNA polymerase
EBV	miR-BART-20-5p	BZLF1	Virus Transactivator
EBV	miR-BART-20-5p	BRLF1	Virus Transactivator
KSHV	miR-K12-9*	RTA	Virus Transactivator
	miR-K12-7-5p		
HCMV	miR-UL112-1	IE72	Major IE gene
MDV-1	miR-M4	UL28	Cleavage/Packaging of Viral DNA
		UL32	Cleavage/Packaging of Viral DNA
MDV-1	miR-M7-5p	ICP4	Virus Transactivator
		ICP27	IE gene (ORF57 homologue)

1.7.3 OvHV-2-encoded miRNAs

As other herpesviruses encode miRNAs it was hypothesised that OvHV-2 encoded miRNAs. High throughput sequencing and bioinformatics analysis of the BJ1035 cell line was performed and from this forty-five miRNAs were predicted (described in further detail in section 3.1, see Figure 3.4 for their location in the OvHV-2 genome) (Levy, 2011). The eight predicted miRNAs with the highest number of reads from the RNA-seq were validated by northern blotting (Levy *et al.*, 2012).

Bioinformatic analysis was used to predict targets within the 5' and 3'UTRs of OvHV-2 ORFs and luciferase assays confirmed the targeting of ORF73 and ORF50 by ovhv2-miR-17-1 and ovhv2-miR-17-10 (Riaz *et al.*, 2014). The targeting of ORF50 is in line with current evidence discussed in section 1.7.2 that virus-encoded miRNAs target IE genes as a mechanism of latency maintenance.

Dr Riaz also performed CLASH in the BJ1035 cell line and in sheep embryo fibroblast cells transduced with a lentivirus containing ovhv2-miR-17-1, miR-17-2 and miR-17-3. Luciferase assays confirmed that *U2* (a component of the spliceosome) was a target of ovhv2-miR-17-28. *DLL1* was also confirmed as a target of ovhv2-miR-17-2 by luciferase assay (see section 4.3 and sections therein for further details) (Riaz, 2014).

1.8 Aims

The identification of OvHV-2-encoded miRNAs is an important step forwards in the understanding of OvHV-2 biology. Validation and target identification of these miRNAs is required to elucidate their roles in virus biology, especially in order to determine whether they play a role in the different outcomes observed between sheep and susceptible species.

The major aims of this thesis were to:

- Validate the remaining thirty-seven predicted miRNAs to confirm whether or not they are expressed by OvHV-2.
- Elucidate the targets of OvHV-2-encoded miRNAs in sheep and cattle by a number of methods including further validation of the CLASH data performed by Dr Riaz (in particular targeting of *DLL1*). Bioinformatic analysis will also be used to predict mRNAs that may be differentially targeted in sheep and cattle.
- Identification and functional characterisation of other viral targets of OvHV-2-encoded miRNAs by bioinformatic analysis.

Chapter 2: Material and Methods

2.1 Tissue Culture

2.2 PCR Methods

2.3 Cloning Methods

2.4 Western Blotting

2.5 Immunoprecipitation

2.6 Flow Cytometry

2.7 Immunofluorescence

2.8 Other Methods

2.1 Tissue Culture

2.1.1 Growth of stable adherent cell lines

HEK-293T (Human embryonic kidney) cells, SEF (sheep embryonic fibroblasts, a gift from Dr Gerry McLaughlin, Roslin Institute) cells, MDBK (Madin-Darby bovine kidney) cells and L929 (mouse aneuploidy fibrosarcoma) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, Gillingham, UK) supplemented with 10% foetal bovine serum (FBS, Life Technologies, Paisley, UK) and 1% penicillin-streptomycin-glutamine (Invitrogen). L929 cells transfected with cosmid vectors expressing the ovine major histocompatibility complex (MHC) class II genes *DRA* and *DRB* (hereafter referred to as LT8.1 cells, Professor John Hopkins, Roslin Institute) were grown in the same conditions as all other cells. Cells were grown in either 75 cm^2 or 175 cm^2 tissue culture flasks (ThermoScientific, Loughborough, UK) in a 37°C, 5% CO_2 incubator.

Confluent cell monolayers were removed from the surface of the flask using Trypsin-EDTA (Ethylenediaminetetraacetic acid, Sigma-Aldrich). The media was poured off and replaced with an equivalent amount of phosphate buffered saline (PBS) to wash the cells. This was removed and a sufficient volume (enough to cover the cell monolayer) of trypsin-EDTA was added and incubated at 37°C, 5% CO_2 until all cells had detached, with the exception of HEK-293T cells which were incubated at room temperature for 1 min. Cells were resuspended in 10 ml of complete DMEM. New tissue culture flasks were seeded at appropriate densities in either 15 ml (75 cm^2) or 25 ml (175 cm^2) of DMEM.

2.1.2 Growth of established cell lines in suspension

BJ1035 cells (a gift from Dr G Russell, Moredun Research Institute) were grown in 25 cm^2 tissue culture flasks in suspension, maintained in Iscove's Modified Dulbecco's Medium (Life Technologies) supplemented with 10% FBS, 1% penicillin-streptomycin, Interleukin-2 (350 IU/ml) (Novartis Pharmaceuticals UK, Camberley, UK) and 0.12% β-mercaptoethanol (Sigma-Aldrich) and incubated at 37°C, 5% CO_2. Growth media was replaced every 2-3 days and once enough large clumps of cells were seen to cover the bottom of the flask the cells were resuspended by pipetting and transferred to two new 25 cm^2 flasks.

2.1.3 Establishment of IL-2 dependent cells

Bovine lymphoblasts were generated by Dr Suzanne Esper from isolated peripheral blood mononuclear cells from the buffy coat of bovine blood and subsequent stimulation with concanavalin A at a concentration of 2.5 µg/ml (Bujdoso *et al.*, 1990). Cells were grown in 25 cm^2 tissue culture flasks and maintained in Roswell Park Memorial Institute (RPMI, Invitrogen) medium supplemented with 10% FBS, 1% penicillin-streptomycin, 0.1% β-mercaptoethanol and 100 U/ml IL-2. Growth media was replaced every 2-3 days. Cells were grown in a 37°C incubator with 5% CO_2.

2.1.4 Preparation of cells for long term storage

Cells were resuspended to 5 x 10^6 cells/ml and pelleted at 500 x g for 5 mins. The supernatant was removed and cells were resuspended in 1 ml of freezing medium (90% FBS, 10% [v/v] dimethyl sulphoxide) and transferred to a cryovial. Samples were wrapped in cotton wool, placed in a Styrofoam box and stored at -80°C overnight. Samples were put in liquid nitrogen the following day for long term storage.

2.1.5 Growth of cell lines from frozen stock

Vials of cells were removed from liquid nitrogen and placed in a 37°C water bath to thaw quickly. The cells were either added to a 25 cm^2 tissue culture flask (suspension cell lines) or a 75 cm^2 flask (adherent cell lines) and an appropriate volume of pre-warmed growth medium was added. Cells were incubated at 37°C, 5% CO_2 overnight and medium changed the following day.

2.1.6 Reverse transfection of cell lines by Lipofectamine 2000

HEK-293T cells were grown in 75 cm^2 flasks until they were 90% confluent. Cells were trypsinised as described in section 2.1.1, counted and resuspended in antibiotic-free DMEM supplemented with 10% FBS at 6 x 10^5 cells/ml. 300 ng of plasmid DNA and 100 nM miRNA mimic (Qiagen, Manchester, UK) were mixed with Opti-MEM (Life Technologies) to a total volume of 25 µl. 1 µl of Lipofectamine 2000 (Life Technologies) and 24 µl of Opti-MEM were mixed and incubated at room temperature for 5 mins. The Lipofectamine 2000-Opti-MEM mixture was added to the DNA-miRNA mimic mixture and complexes were allowed to form by incubating at room temperature for 15 mins. 100 µl of HEK-293T cells at 6 x 10^5 cells/ml were added to the complexes and the total 150 µl volume was transferred to one well of a 96 well plate. Cells were incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

2.1.7 Transfection of cell lines by Lipofectamine 2000

HEK-293T cells were seeded at 7.5 x 10^5 cells per well of a 6 well plate in DMEM supplemented with 10% FBS the day before transfection. Before seeding the cells the wells were treated with poly-L-lysine (Sigma). 400 µl of poly-L-lysine was added to each well and incubated for 5 mins at room temperature. The poly-L-lysine was aspirated from the wells and the wells allowed to air-dry before seeding cells. 2.5 µg of plasmid DNA was complexed with 10 µl of Lipofectamine 2000 in Opti-MEM to a total volume of 300 µl as described in section 2.1.6. Growth media was removed from the cell monolayers and replaced with 700 µl of DMEM supplemented with 10% FBS. The total 300 µl of Lipofectamine 2000-DNA mixture was added to the cells and incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

The day before transfection 2 x 10^5 HEK-293T cells were seeded in one well of a 12 well plate in DMEM supplemented with 10% FBS. 100 ng of plasmid DNA and 100 nM miRNA mimic were complexed with 5 µl of Lipofectamine 2000 in Opti-MEM to a total volume of 100 µl as described in section 2.1.6. The total 100 µl of Lipofectamine 2000-DNA-miRNA mimic complexes were added to the cells and incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

For transfections in 96 well plates, HEK-293T cells were seeded the day before at 3 x 10^4 cells per well in DMEM supplemented with 10% FBS. 10 ng of plasmid DNA and 100 nM miRNA mimic were complexed with 0.5 µl of Lipofectamine 2000 in Opti-MEM to a total volume of 10 µl as described in section 2.1.6. The total 10 µl Lipofectamine 2000-DNA-miRNA mimic complexes were added to the cells and incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

2.1.8 Transfection onto coverslips

The wells of a 24 well plate were treated with 200 µl poly-L-lysine as described above. One coverslip (12 mm in diameter, Scientific Laboratory Supplies, Coatbridge, UK) was placed into one well of a 24 well plate. Coverslips were sterilised in 1 ml Ethanol. This was removed and coverslips were allowed to air-dry. HEK-293T cells were seeded at 1.5 x 10^5 cells per well in DMEM supplemented with 10% FBS the day before transfection. 500 ng of plasmid DNA was complexed with 1 µl of Lipofectamine 2000 in Opti-MEM to a total volume of 50 µl as described in section 2.1.6. The total 50 µl of Lipofectamine 2000-DNA mixture was added to the cells and incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

2.1.9 Transfection of cell lines by nucleofection

MDBK cells were grown in 75 cm^2 flasks until they were 90% confluent. Cells were trypsinised as described in section 2.1.1, counted and resuspended in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine at 1 x 10^6 cells per sample. Cells were spun at 500 x g for 5 mins at room temperature. The supernatant was completely removed from the pellet and cells were resuspended in 100 µl of room temperature Nucleofector® Solution (part of the Cell Line Nucleofector® kit R, Lonza, Slough, UK) per sample. 100 µl cell suspension was combined with 2 µg of plasmid DNA and transferred into the supplied cuvettes, and the cap placed on the cuvette. The cuvette was placed into the Nucleofector II (Lonza), programme X-001 was selected and the programme was applied. The cuvette was removed from the holder and incubated for 10 mins at room temperature. Following incubation, 500 µl of DMEM supplemented with 10% FBS and penicillin-streptomycin-glutamine was added and samples were immediately transferred to prepared 6 well plates using the supplied pipettes. 6 well plates were prepared by adding 1 ml of DMEM supplemented with 10% FBS and penicillin-streptomycin-glutamine and incubating at 37°C with 5% CO_2. Cells were incubated for 48 hrs at 37°C with 5% CO_2 before harvesting.

Table 2.1 Summary of Transfection Protocols

Transfection	Cell Line	Plate Size	Seeding Density (per well)	Lipofectamine 2000 (µl)	Plasmid DNA (µg)	miRNA mimic (nM)
Forward	HEK-293T	6	7.5 x 10^5	10	2.5	n/a
		12	2 x 10^5	5	0.1	100
		24	1.5 x 10^5	1	0.5	n/a
		96	3 x 10^4	0.5	0.001	100
Reverse	HEK-293T	96	6 x 10^4	1	0.3	100

Described in further details in sections 2.1.6, 2.1.7 and 2.1.8.

2.2 PCR methods

2.2.1 DNA Isolation

Total DNA was isolated from a maximum of 5 x 10^6 cells per sample using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was determined using a NanoDrop ND-100 Spectrophotometer (Thermo Scientific) and stored at -20°C.

2.2.2 RNA Isolation

Total RNA was isolated from a maximum of 1 x 10^7 cells per sample using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was determined using a NanoDrop ND-100 Spectrophotometer and stored at -80°C.

2.2.3 Reverse Transcription of RNA

Before reverse transcription (RT), DNase treatment of RNA was performed using the TURBO DNA-free™ Kit (Life Technologies). 1 µg of RNA was treated in a 50 µl reaction volume according to the manufacturer's protocol. 10 µl of DNase-treated RNA was incubated with 1 µl of either random heaxamer primers or oligo(dT) primers (both Promega, Southampton, UK) at 70°C for 5 mins before placing on ice. 30 U Avian Myeloblastosis Virus Reverse Transcriptase (Promega), 2.5 µl 10 mM dNTPs (Bioline, London, UK) and 5 µl supplied buffer were added to the DNase-treated RNA and primer mix to a total volume of 25 µl and incubated at either 37°C (random hexamer primers) or 42°C (oligo(dT)) primers for 1 hr. cDNA concentration was determined using a NanoDrop ND-100 Spectrophotometer and stored at -20°C.

2.2.4 Reverse Transcription of small RNAs

Reverse transcription was performed using the miScript II RT Kit (Qiagen). 200 ng of RNA was used in a total reaction volume of 10 µl with the miScript HiSpec Buffer (supplied with miScript II RT Kit) according to the manufacturer's protocol.

miRNA-specific hairpin-loop reverse transcription primers were designed as described in (Varkonyi-Gasic & Hellens, 2011) and were supplied by Eurofins Genomics (Ebersberg, Germany). A reverse transcription reaction containing 25 µl 200 µM stock of reverse transcription (RT) primer, and 10 x RT Omniscript buffer (Qiagen) to a total volume of 100 µl was used to anneal the RT primer. Conditions for annealing were as follows: 95°C 30 for mins, 72°C for 2 mins, 37°C for 2 mins and 25°C for 2 mins. RT primer concentration after

annealing was 50 µM and working concentration was 5 µM. A list of RT primers can be found in Appendix 2.

RT reactions contained 200 ng RNA in a total reaction volume of 15 µl with 10 x RT Omniscript buffer, 1.5 pmols RT primer, 0.15 µl 10 mM dNTPs ,0.19 µl RNaisin (Promega) and 1 µl Multiscribe Reverse Transcriptase (Life Technologies). Reverse transcription cycling conditions were as follows: 16°C 30 mins, 42°C 30 mins, 85°C 5 mins. cDNA was stored at -20°C.

2.2.5 Polymerase Chain Reaction

All Polymerase chain reactions (PCR) (and subsequent PCRs herein with the exception of sections 2.2.6 and 2.2.10) were carried out in a Veriti 96-well thermal cycler (Life Technologies). primer3 (http://primer3.ut.ee/) was used for primer design. A total reaction volume of 20 µl contained 1 - 2 µl cDNA template, 8 pmols forward and reverse primers, 1 u HotStarTaq DNA Polymerase (Qiagen), 0.4 µl dNTPS (10 mM) and 2 µl 10 x PCR Buffer (supplied with HotStarTaq DNA Polymerase). Cycling conditions were as follows: 95°C for 5 mins, 35 cycles of 95°C for 30 sec, 55-60°C (depending on T_m of primers) for 1 min, 72°C for 1 min followed by a final 72°C for 7 mins.

2.2.6 PCR of cDNA made using the Qiagen miScript Reverse Transcription Kit

PCR was performed using the miScript SYBR Green PCR Kit (Qiagen). A 20 µl total reaction volume in 0.1 ml thin-walled PCR tubes (Qiagen) was set up containing 500 ng cDNA, 10 pmols specific forward primer (Life Technologies), 10 pmols universal reverse primer and 10 µl 2 x SYBR green (both supplied in miScript SYBR Green PCR Kit). PCR cycling conditions were as recommended by the manufacturer's protocol, with the exception of the annealing temperature which changed in accordance with the T_m of the forward primer. PCR was carried out in either a Rotor-Gene Q or Rotor-Gene RG-3000 (both Qiagen). A list of lists forward primer sequences and annealing temperatures can be found in Appendix 2.

2.2.7 PCR of miRNA-specific cDNA

PCR carried out using miRNA-specific cDNA was performed using the PCR protocol in section 2.2.5 with the forward and reverse primer concentration increased to 12 pmol. Cycling conditions were as follows: 95°C for 5 mins, 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, followed by 72°C for 7 mins. A list of forward primers and the universal reverse primer can be found in Appendix 2.

2.2.8 Site-directed Mutagenesis PCR

Site-directed mutagenesis PCR was carried out using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Manchester, UK). Mutagenesis primers were designed using the QuikChange Primer Design Program (www.agilent.com/genomics/qcpd) and are listed in Appendix 2. Reactions, cycling parameters and *Dpn I* restriction enzyme digestion were carried out according to the manufacturer's protocol.

2.2.9 Rapid Amplification of cDNA ends PCR

Rapid Amplification of cDNA ends (RACE)-ready sheep mesenteric lymph node PCR product was a kind gift from Louise Nicol. RACE PCR was performed using the GeneRacer Kit (Life Technologies). Gene-specific primers were designed according to the manufacturer's protocol and are listed in Appendix 2. PCR reactions were set up using PlatinumTaq (Life Technologies) according to the manufacturer's protocol except for 50 mM $MgSO_4$ which was replaced with 50 mM $MgCl_2$. Cycling parameters were performed according to the manufacturer's protocol.

2.2.10 Quantitative PCR

Quantitative PCR (RT-qPCR) was carried out in either a Rotor-Gene Q or Rotor-Gene RG-3000. SensiFAST SYBR Hi-ROX Kit (Bioline) was used for amplification. 20 µl total reaction volumes were set up according to the manufacturer's protocol using 500 ng cDNA. primer3 (http://primer3.ut.ee/) was used for primer design of amplicons no more than 200 bp in length (see Appendix 2 for a list of primers). 3-step cycling parameters were performed according to the manufacturer's protocol. Samples for RT-qPCRs described in section 5.4.2 were set up with the aid of the CAS-1200 Liquid Handling System (Qiagen).

2.2.11 Agarose Gel Electrophoresis

DNA was analysed by electrophoresis in 0.8 – 3% agarose gels containing 1x SYBR® Safe DNA Gel Stain (Life Technologies) in Tris-acetate buffer (TAE) buffer. Samples up to 15 µl were mixed with 5 x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 0.1 M EDTA, 30% glycerol in H_2O). Electrophoresis was carried out in tanks containing TAE buffer at 80-90 V for 50 ml gels and 80 – 100 V for 100 ml gels. Estimation of DNA size was performed by comparison to 1 kb, 100 bp or 50 bp GeneRuler DNA Ladders (Thermo Scientific). Gels were visualised on a UV transilluminator.

2.3 Cloning Methods

2.3.1 Purification of PCR products

PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, UK) according to the manufacturer's protocol.

2.3.2 Annealing of Oligonucleotides for Cloning

Oligonucleotides up to 100 bases in length were synthesised along with their reverse complement counterpart by either Invitrogen or Sigma (see Appendix 2 for a list of oligonucleotides). Oligonucleotides were resuspended to 100 µM. 1 µl of each pair of oligonucleotides was mixed with 5 µl of 10 x Buffer 2 (supplied with New England Biolabs [NEB] Restriction Endonucleases) to a total volume of 50 µl. The sample was heated at 95°C for 5 minutes in a heat block which was then turned off and allowed to cool to room temperature. Annealed oligonucleotides were then treated with T4 Polynucleotide Kinase (NEB, Hitchin, UK). 6 µl T4 DNA Ligase buffer (NEB) and 4 µl T4 Polynucleotide Kinase were added to 50 µl of annealed oligonucleotides and incubated at 37°C for 30 mins. Ligations with annealed oligonucleotides could then be performed into vectors cut with appropriate restriction endonucleases. Oligonucleotides longer than 100 bases in length were synthesised as dsDNA by Integrated DNA Technologies (Leuven, Belgium) and required digestion with appropriate restriction endonucleases before ligation.

2.3.4 DNA Ligation

When PCR products only required sequencing the TOPO® TA Cloning® Kit (Life Technologies) was used for ligation. 4 µl PCR purified product was added to 1 µl Salt solution and 1 µl TOPO 2.1 vector and left for 15 mins at room temperature.

For cloning DNA digested with appropriate restriction endonucleases into expression vectors (see appendix 1 for vector maps) the LigaFast™ Rapid DNA Ligation System (Promega) was used. A ratio of 1:3 of vector to insert DNA was used for cloning with 3 u of T4 DNA Ligase (supplied in the LigaFast™ kit) in a final total of 10 µl. Ligations were incubated for 15 mins at room temperature before transformation.

2.3.5 Transformation of One-Shot Chemically Competent Cells

5 µl ligated PCR product was added to 25 µl TOP10 chemically competent cells (Life Technologies) and left on ice for 30 mins. Samples were heat shocked at 42°C for exactly 45 sec and placed back on ice for 2 mins. 250 µl SOC medium (Life Technologies) was added and cells placed in a shaking incubator at 200 rpm for 1 hr at 37°C. The cells were spread on

Luria-Bertani (LB) plates supplemented with appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) and incubated at 37°C overnight.

2.3.6 Transformation of XL-10 Gold Ultracompetent Cells

2 μl of β-Mercaptoethanol was added to 45 μl XL-10 Gold Ultracompetent cells (supplied with QuikChange Lightning Site-Directed Mutagenesis Kit) and incubated on ice for 10 mins with occasional swirling. 2 μl of ligated product was added to the cells and incubated for 30 mins on ice. Cells were then heatshocked at 42°C for exactly 30 sec and placed back on ice for 2 mins. 400 μl SOC medium was added and cells placed in a shaking incubator at 200 rpm for 1 hr at 37°C. The cells were spread on Luria-Bertani (LB)/Agar plates supplemented with appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) and incubated at 37°C overnight.

2.3.7 Preparation of Bacterial stocks for long term storage

A single bacterial colony was picked from LB/Agar plates and used to inoculate 4 ml of LB medium supplemented with the appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin). Cultures were shaken overnight at 200 rpm in a shaker at 37°C. 500 μl of culture was added to 500 μl of sterile 80% glycerol in H_20 and mixed thoroughly by inversion. Samples were stored at -80°C.

2.3.8 Plasmid DNA Isolation from Bacteria (Small Scale)

A single bacterial colony was isolated from LB/Agar plates and used to inoculate 4 ml of LB medium supplemented with the appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin). Cultures were shaken overnight at 200 rpm in a shaker at 37°C. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol and resuspended in milli-Q H_2O (Millipore, Watford, UK). The concentration of purified plasmids was determined using a NanoDrop as in section 2.2.1. Plasmids were stored at -20°C.

2.3.9 Plasmid DNA Isolation from Bacteria (Large Scale)

100 ml of LB medium supplemented with the appropriate antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) was inoculated with 100 μl of culture set up as in section 2.3.8. Cultures were shaken overnight at 200 rpm in a shaker at 37°C. Plasmids were isolated using the Qiagen Plasmid Midi Kit (Qiagen) according to the manufacturer's protocol and resuspended in milli-Q H_2O. The concentration of purified plasmids was determined using a NanoDrop as in section 2.2.1. Plasmids were stored at -20°C.

2.3.10 Restriction Digest of DNA

DNA was digested with restriction endonucleases (*EcoRI*, *XhoI*, *NotI*, *KpnI*, *BamHI* and *HindIII*, NEB) according to manufacturer's instructions using the supplied buffers. Restriction digests were incubated at 37°C for 1 hr in a total volume of 25 µl. When performing restriction digests for cloning purposes, 2 µg of vector DNA was incubated with 2 U of the appropriate restriction endonucleases in a total volume of 50 µl at 37°C for 1 hr. Digested samples were analysed on a 0.8% agarose gel. Bands were excised from the gel with a clean sharp scalpel blade and DNA purified as described in section 2.3.1. The concentration of vector DNA was determined before treating the cut vector with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) according to the manufacturer's protocol.

2.3.11 Sequencing of Plasmid DNA

Plasmid DNA was sent for sequencing at GATC biotech (London, UK) according to their instructions.

2.3.12 Sequence Analysis

DNA Sequences were analysed using NCBI nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast) and Ensembl BLAT (http://www.ensembl.org/Multi/Tools/Blast). NEBcutter (http://tools.neb.com/NEBcutter2/) was used for restriction digest mapping.

2.4 Western Blotting

2.4.1 Protein Sample Preparation

Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) was prepared by adding 50 µl of β-Mercaptoethanol to 950 µl Laemmli sample buffer. The supernatant of transfected cell monolayers was removed and a minimum of 200 µl of prepared Laemmli sample buffer was added to each well of a 12 well dish. Samples were mixed thoroughly with a pipette to ensure proper lysis before being transferred to a 1.5 ml microcentrifuge tube. Samples were boiled for 5 minutes at 95^{o}C before loading onto an SDS-PAGE gel.

2.4.2 SDS-polyacrylamide gel electrophoresis

4-20% Mini-PROTEAN® TGX™ Gels (Bio-Rad) were used for SDS-PAGE. 1x Tris/Glycine/SDS running buffer was prepared by mixing 10x Tris/Glycine/SDS (Bio-Rad) with H_2O. Samples were loaded into wells and run at 120 V for 1 – 1.5 hrs or until the blue dye front had reached the bottom of the gel. The Odyssey® Protein Molecular Weight Marker (LI-COR) was used to determine approximate protein size.

2.4.3 Transfer of Protein to Nitrocellulose Membranes

Transfer buffer was prepared with 20% v/v methanol in 1x Novex® Tris-Glycine Transfer Buffer (Life Technologies) to 1x in H_2O. Nitrocellulose membrane and filter paper (Nitrocellulose/Filter Paper Sandwich, 0.45 µm pore size, 8.5 cm x 13.5 cm, Life Technologies) were cut to size and soaked in transfer buffer before assembly of transfer cassette. A sponge pad was laid on the black side of the cassette followed by 1 piece of filter paper, protein gel, nitrocellulose membrane, 1 piece of filter paper and a sponge pad. The cassette was closed and inserted into the transfer unit. Transfer was run at 100 V for 1 hr. 4% w/v Amersham ECL Prime Blocking Reagent (GE Healthcare Sciences) in PBS-T (PBS with 0.1% v/v Tween 20 [Sigma-Aldrich]) was used and the nitrocellulose membrane was incubated with rocking at room temperature for 1 hr, followed by 3 x 5 min washes in PBS-T.

2.4.4 Immunological Staining of Protein Blots

Membranes were incubated with primary antibodies (Table 2.2). All antibodies were used at the dilution suggested by the manufacturer in PBS-T. A rabbit Anti-Ov2 Antibody was synthesised using EKK and RRS peptides by Dundee Cell Products (Dundee, UK) and was used at a dilution of 1:1000 in PBS-T. Primary antibody incubations were carried out with rocking at 4^{o}C overnight. Three x 5 min washes in PBS-T were performed before incubation with secondary antibodies (LI-COR Biotechnology, Cambridge, UK, Table 2.2). Secondary antibodies were diluted 1:10000 in PBS-T and membranes were incubated with rocking at

room temperature for 45 mins before a further three x 5 min washes in PBS-T. Membranes were visualised on the Odyssey CLx (LI-COR Biotechnology).

2.4.5 Quantitative Analysis of Protein Blots

Analysis of protein blots was performed using Image Studio Lite version 3.1 (LI-COR Biotechnology). Protein levels were quantified by determining the signal of each band and normalising the protein band of interest to a housekeeping gene.

Table 2.2. Antibodies and their appropriate dilutions

Primary Antibodies	**Dilution**	**Use**	**Manufacturer**
Rabbit anti-Ov2	1:1000	WB/IF	Dundee Cell Products
Mouse anti-Actin	1:1000	WB	Abcam (Cambridge, UK)
Rat anti-MHC class II (SW73.2)	1:1000	FC	Kind gift from Professor John Hopkins (Hopkins *et al.*, 1986)
Rat IgG	1:500	FC	Kind gift from Professor John Hopkins
Rat anti-HA	1:1000	IF	Roche (Burgess Hill, UK)
Secondary Antibodies	**Dilution**	**Use**	**Manufacturer**
IRDye 680RD Donkey Anti-Mouse IgG	1:10000	WB	LI-COR
IRDye 800CW Donkey Anti-Rabbit	1:10000	WB	LI-COR
Anti-Rat IgG Alexa Fluor 488	1:1000	FC	Cell Signalling Technology (Leiden, Netherlands)
Anti-Rat IgG Alexa Fluor 555	1:1000	IF	Cell Signalling Technology
Anti-Rabbit IgG Alexa Fluor 488	1:1000	IF	Cell Signalling Technology

WB = western blotting. FC = flow cytometry. IF = immunofluorescence.

2.5 Immunoprecipitation

2.5.1 Cell Lysate Preparation

The supernatant of transfected cell monolayers was removed and 500 µl ice cold PBS added to each well. The PBS was removed and 400 µl of ice cold lysis buffer (25 mM Tris HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 5% glycerol) supplemented with 1 mM phenylmethanesulfonylfluoride (PMSF) and 1:100 protease inhibitor cocktail (both Active Motif, La Hulpe, Belgium) was added. Cells were incubated on ice for 15 mins. Samples were mixed thoroughly with a pipette to ensure proper lysis before being transferred to a 1.5 ml microcentrifuge tube. Samples were spun at 15000 x g for 15 mins. The supernatant was transferred to a clean microcentrifuge tube and lysates were stored at -80 ^{o}C if they were not used immediately.

2.5.2 Immunoprecipitation

Immunoprecipitation (IP) was performed using Pierce Anti-HA Magnetic beads (Thermo Scientific). A magnetic stand was used to separate the beads. 25 µl of bead slurry was used per sample. Beads were washed twice in Tris-buffered saline with Tween (TBS-T, 0.05% Tween, Thermo Scientific) before adding the cell lysate. Samples were incubated for 30 mins at room temperature with rotation. The beads were separated and the unbound sample removed and stored at -20 ^{o}C. The beads were thoroughly suspended in 300 µl of TBS-T, magnetically separated and the supernatant removed three times before a final wash in 300 µl of ultrapure water. For samples to be eluted from the beads to confirm that the immunoprecipitation was successful, the beads were magnetically separated, the supernatant was removed and 100 µl of laemmli sample buffer was added. Samples were stored at -20 ^{o}C until further use. Confirmation of the presence of the protein of interest was confirmed by western blotting as described in sections 2.4.2 to 2.4.5. For samples subsequently analysed by mass spectrometry, the beads were magnetically separated, the supernatant was removed and the beads stored at -20 ^{o}C until further use. These were processed by the Proteomics and Metabolomics Facility at the Roslin Institute using on-bead tryptic digestion.

2.5.3 Mass Spectrometry

Liquid chromatography tandem mass spectrometry (LC-MS/MS) and data analysis were carried out by the Proteomics and Metabolomics Facility at the Roslin Institute. LC-MS/MS was performed by coupling a RSLCnano LC system (Thermo Scientific) to a micrOTOF-II mass spectrometer (Bruker, Germany). Spectra were deconvoluted and the peak lists exported as Mascot Generic Files (MGF) and searched using Mascot 2.4 server (Matrix Science, London, UK) against the Uniprot Human sequence database appended with the Ov2 sequence (entry Q2VSN7). The search was performed using the following parameters: MS tolerance = ±25 ppm, MS/MS tolerance = ±0.1 Da and peptide charge 2+ and 3+. False discovery rate was limited to < 1% for peptide IDs after searching reverse databases.

2.6 Flow Cytometry

2.6.1 Staining of Cells

Cells were trypsinised as described in 2.1.1. Cells were pelleted at 500 x g for 5 mins, counted and resuspended at 2 x 10^7 cells/ml in PBS with 0.5% w/v bovine serum albumin (BSA, Thermo Scientific), 2% v/v FBS and 2 nM EDTA (Thermo Scientific). 1 x 10^6 cells were added to each well in a 96 well plate. An equal volume of primary antibody (Table 2.2) was added and incubated at 4°C for 30 mins. Rat IgG was used as an isotype control. Cells were spun at 500 x g for 5 mins and washed in PBS/BSA/FBS/EDTA solution 3 times. Cells were resuspended in 100 µl of secondary antibody (Table 2.2) at 4°C for 30 mins in the dark. Cells were washed in PBS/BSA/FBS/EDTA solution 3 times and transferred to FACS tubes (BD Biosciences, Oxford, UK) in a total volume of 400 µl PBS/BSA/FBS/EDTA solution.

2.6.2 Harvesting of Cells Transfected with GFP Constructs

Growth Medium was removed from the cells and 50 µl Tryspin-EDTA added to each well. 50 µl growth medium was added and cells were resuspended and transferred to a 96-well u bottom plate. Cells were pelleted at 500 x g for 5 mins. Cells were washed in PBS with 1% w/v BSA and pelleted at 500 x g for 5 mins before being resuspended in 200 µl PBS with 1% w/v BSA. Cells were stored at 4°C until used.

2.6.3 Flow Cytometry using FACSCalibur

A FACSCalibur (BD Biosciences) was used to analyse cells stained as described in section 2.6.1. Forward scatter (cell size) and side scatter (cell granularity) were used to gate live cells. 10,000 live cells were counted and FL1 fluorescence was used to measure MHC class II positive cells.

2.6.4 Flow Cytometry using Fortessa High Throughput Sampler

An LSRFortessa High Throughput Sampler (BD Biosciences) was used to analyse 96 well plates with cells transfected with GFP constructs. Forward scatter (cell size) and side scatter (cell granularity) were used to gate live cells. The 530/30 (FL1) filter was used to detect and count 7500 GFP-positive cells. Cell doublets were removed from data analysis using forward scatter area over height.

2.6.5 Flow Cytometry Analysis

Flow Cytometry data was analysed using version 10.0.6 of FlowJo. Median Fluorescence Intensity (MFI) was used as a measure of knockdown of genes of interest by miRNAs. Statistical analysis was performed on the MFI values as described in section 2.6.3.

2.7 Immunofluorescence

2.7.1 Fixing and Immunostaining of Cells

The supernatant of transfected cell monolayers on coverslips was removed and 1 ml of PBS added to each well. The PBS was removed and 1 ml of Acetone/Methanol (1:1 v/v) was added. Coverslips were incubated on ice for 10 mins. This was removed and the coverslips were washed in PBS three times. Coverslips were incubated for 10 mins at room temperature in 10 mM Ammonium Chloride and the subsequently washed in PBS three times. Coverslips were blocked for 30 mins in 3% v/v Normal Goat Serum (NGS, Cell Signalling Technology)/PBS at room temperature. Primary antibodies (Table 2.2) were diluted in 3% NGS/PBS and coverslips were incubated in the primary antibody for 1 hr at room temperature. Three washes in PBS were performed before adding the secondary antibodies (Table 2.2, also diluted in 3% NGS/PBS). Coverslips were incubated for 45 mins at room temperature in the dark. Three washes in PBS were performed before staining with 4',6-diamidino-2-phenylindole (DAPI, Cell Signalling Technology), diluted 1:1000 in 3% NGS/PBS. Coverslips were incubated with DAPI for 5 mins at room temperature in the dark before three washes in PBS and one wash in distilled water were performed. Coverslips were mounted onto superfrost slides (Thermo Scientific) with ProLong Gold antifade (Invitrogen) and cured overnight at room temperature in the dark. Coverslips were sealed onto the slide the following day with clear nail varnish and were stored at 4^{o}C until imaged.

2.7.2 Confocal Microscopy

Slides were imaged on a Zeiss LSM 710 confocal microscope at the Roslin Institute Bio-Imaging unit with the help of Mr. Bob Fleming. Image analysis was performed using ZEN Lite software.

2.8 Other Methods

2.8.1 Luciferase Assays

Luciferase assays were carried out using the Dual Luciferase Reporter Assay System (Promega). Cells were transfected as in section 2.1.6. Passive lysis of cells in 96 well plates was carried out according to the manufacturer's protocol except that the cells were not washed in PBS before addition of passive lysis buffer and cells were incubated with rocking for 30 mins. Luciferase Assay Reagent II (LAR II) and Stop & Glo Reagent were prepared according to the manufacturer's protocol but was used at a dilution of 1:10. 50 µl of both LAR II and Stop & Glo Reagent were used per reaction instead of the suggested 100 µl. 10 µl of cell lysate was transferred to a 96 well white plate (Sigma-Aldrich) and luciferase assay activity was read using the GlowMax Multi Detection System (Promega).

2.8.2 Statistical Analysis

Statistical analysis was performed using Minitab 17 software. Graphs were made using the Graphpad Prism 6 software. All error bars on graphs represent standard error of the mean unless otherwise stated. Following advice from Dr. Helen Brown, senior statistician at the Roslin Institute differences between scrambled siRNA and miRNA mimics were analysed using a general linear model followed by a post-hoc Tukey's analysis. P values represent results from the post-hoc test. This method of analysis was used for all luciferase assays, GFP flow cytometry assays and Western Blotting performed in subsequent sections. RT-qPCRs described in section 5.4.2 were analysed using the ΔΔCt method (Livak & Schmittgen, 2001).

Commonly Used Solutions

PBS

50 x TAE buffer

LB medium

LB/Agar

All solutions provided by the Central Services Unit at the Roslin Institute.

Chapter 3: Validation of OvHV-2-encoded miRNAs

3.1: Introduction

3.2: Aims

3.3: RT-PCR using the miScript Kits

3.4: Specific cDNA method

3.5: Discussion

3.1 Introduction

To investigate whether OvHV-2 encoded miRNAs, massively parallel sequencing of small RNAs from the OvHV-2 immortalised bovine LGL cell line BJ1035 was performed previously by Dr Levy. Reads were mapped to the virus genome and grouped together based on their genomic position (Levy *et al.*, 2012). Groups were subjected to secondary structure analysis to confirm whether they represented putative miRNAs. Criteria used for this analysis included a stem loop of 60 – 110 nucleotides recognised by the enzyme Drosha; 3' overhangs in the miRNA/miRNA* duplex due to cleavage by Drosha and another miRNA processing enzyme Dicer; and ≤ 16 base pairs within the miRNA/miRNA* duplex required for stability. Forty-five groups were found to fit these criteria, forty-two on the minus-strand and three on the plus-strand of the virus genome. The eight groups with the highest number of reads from the small RNA sequencing data were validated by Dr Levy by northern hybridisation and were named ovhv2-miR-1 to -8.

As the remaining thirty-seven miRNAs were only predictions, confirmation that these are miRNAs encoded by OvHV-2 was necessary. A PCR strategy for validation of the remaining candidate miRNAs was adopted for a number of reasons. Firstly, PCR is a more amenable method for a larger number of samples than northern hybridisation. Secondly, PCR also allows for possible quantification of miRNA transcripts unlike northern hybridisation which is a non-quantitative method. A quantitative system could be particularly useful in an animal model of MCF and provide insight into what roles virus-encoded miRNAs play during infection. Finally, the number of reads from the small RNA sequencing data ranged from 1 to 46048 (see Table 3.1) and consequently a more sensitive approach such as PCR was needed.

The following sections describe two PCR methods used to validate the expression of OvHV-2-encoded miRNAs.

3.2 Aims

The aim of this part of the project was to validate the expression of the remaining predicted OvHV-2-encoded miRNAs by PCR.

3.3 RT-PCR using the miScript Kit

RNA was extracted from BJ1035 cells and reverse transcription performed as in sections 2.2.2 and 2.2.4. Previous work had used the *Theileria parva*-immortalised bovine T-cell line 495TPM as a negative control however this was not available for use when the PCR validation work was carried out. Instead, bovine lymphoblasts generated as in section 2.1.3 were used as the negative control cell line throughout the validation of OvHV2-encoded miRNAs by PCR methods. miRNA-specific forward primers were designed using the first ~16 nucleotides of the predicted miRNA sequence so that resulting products could be cloned and sequenced. PCRs were performed in triplicate using the miScript SYBR Green PCR Kit as described in section 2.2.6 and analysed by gel electrophoresis. Due to the polyadenylation step and use of an oligo-dT primer with a 3' universal tag during reverse transcription genomic DNA is not detected during the PCR and so minus reverse transcription (minus RT) controls were not necessary.

Resulting products are shown in Figure 3.2 for thirty-three out of thirty-seven groups analysed. No amplification by RT-PCR was observed for two groups (1 and 3p) and so were not analysed by agarose gel electrophoresis.

Group 217 (renamed ovhv2-miR-73-1) displays 5' homology to the mammalian miRNA miR-216a (Figure 3.1) and was not analysed by this first PCR method. Group 67 is the only miRNA confirmed by sequencing for which a gel image is absent. According to the Qiagen miScript PCR System Handbook the expected PCR product size of mature miRNAs should be 85-87 bp. The majority of groups (with the exception of groups 46, 95, 163, 64p and 96p) displayed a product of between 50 and 100 bp which fits with the expected product size. Group 46 had a product size of approximately 125 bp and group 96p had a product size of approximately 150 bp. Product was only observed in BJ1035 samples for group 46. This larger product size could represent a pre-miRNA form however this is unlikely due to the priming of the cDNA during the PCR. Upon cloning and subsequent restriction digest a band of the correct size was observed. It is possible that a product of the correct size was generated but not discerned by agarose gel electrophoresis and as this product is smaller it would be more efficiently cloned. The product observed for group 96p could represent off-target priming of cellular cDNA as a band of the same size is also observed in the bovine lymphoblast samples. A second band of approximately 100 bp and 125 bp was observed for groups 181 and 182 respectively. The larger size product could again represent off-target amplification as the band can be seen in both BJ1035 and bovine lymphoblast samples.

Any sample that showed a band regardless of size was cloned and sent for sequencing. As no distinct band could be discerned for groups 95, 163 and 64p they were not sequenced. Twenty-two out of thirty-two groups sequenced were confirmed to be miRNAs using this method. When the remaining nine groups (3, 13, 45, 53, 60, 128, 181, 182, 96p) were sequenced the resulting sequence contained either the primer sequence followed by a few nucleotides that did not match the predicted miRNA sequence or the primer sequence followed by a number of nucleotides that aligned to a bovine mRNA sequence. This most likely represents off-target effects of the primers which could not be improved due to the constriction of primer design. These latter results were similar to the sequences obtained when any bovine lymphoblast samples were sequenced and as such could not be confirmed by this method.

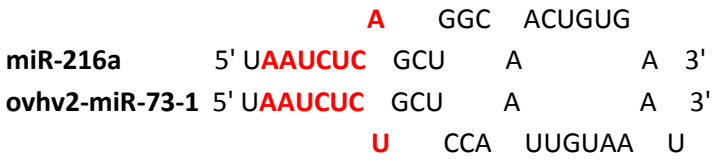
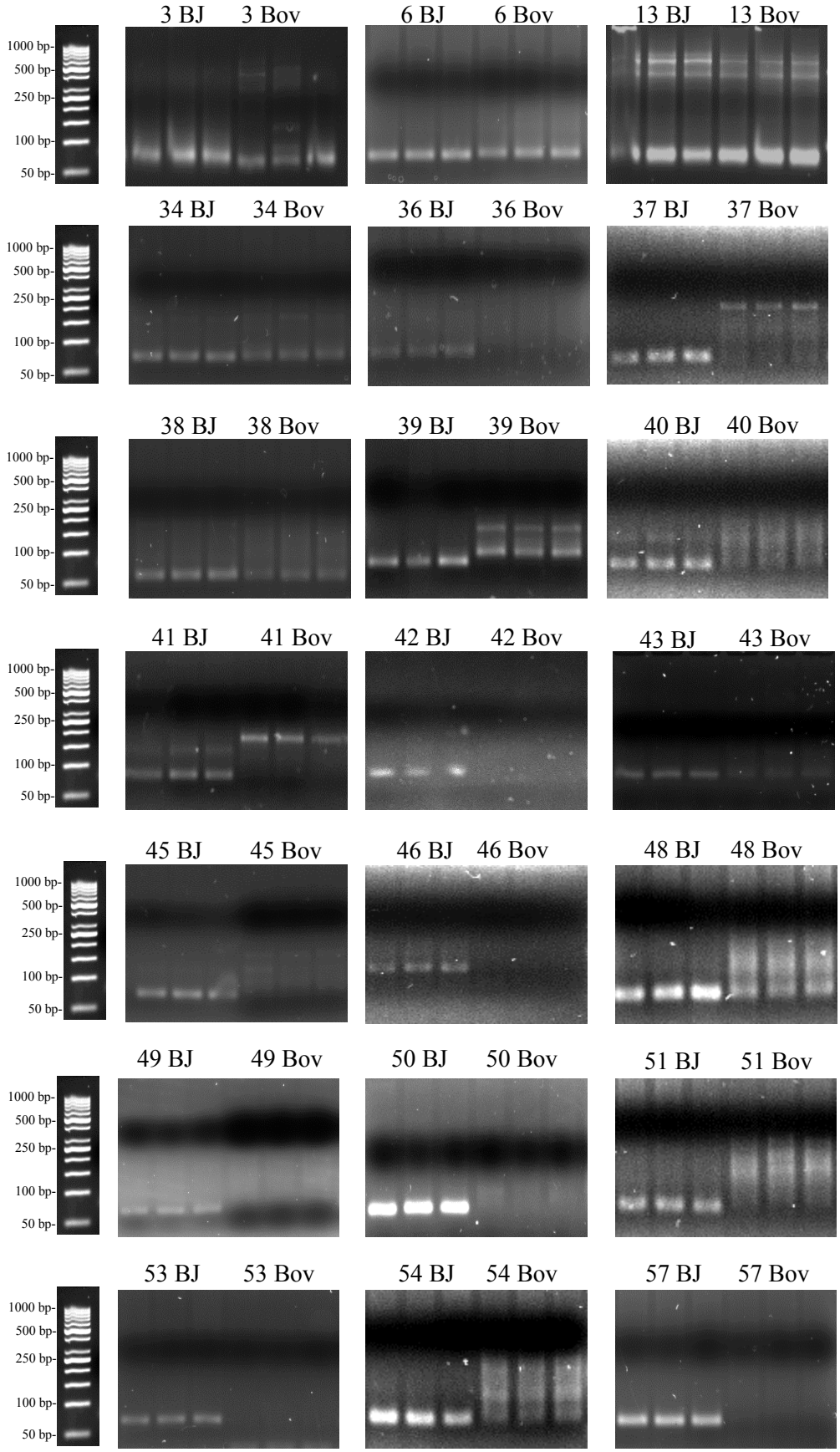


Figure 3.1. Comparison of the sequences of ovhv2-miR-73-1 and miR-216a

Seed sequences are shown in red.


3 BJ
3 Bov
6 BJ
6 Bov
13 BJ
13 Bov
34 BJ
34 Bov
36 BJ
36 Bov
37 BJ
37 Bov
38 BJ
38 Bov
39 BJ
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500 bp-
250 bp-
100 bp-
50 bp-

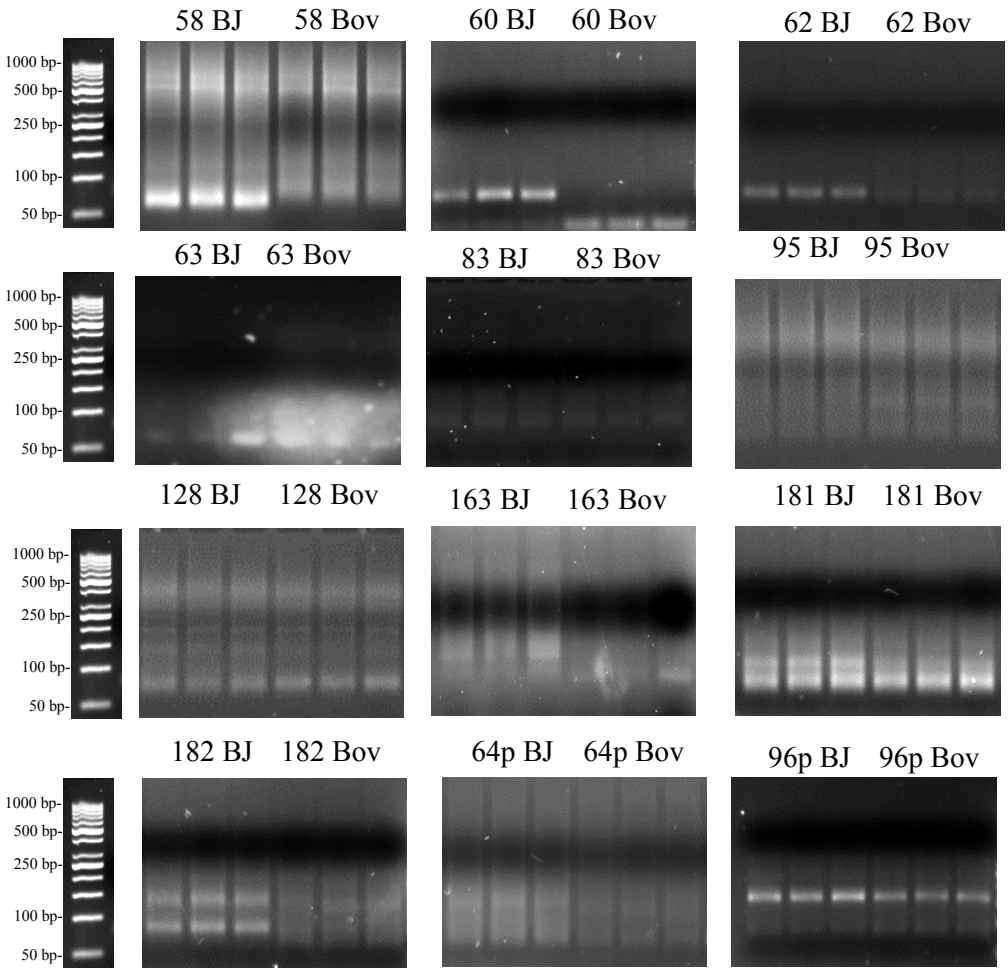


Figure 3.2. Analysis of OvHV-2-encoded miRNA expression using the miScript Kits

RT-PCRs for candidate miRNAs were analysed by agarose gel electrophoresis. Numbers represent groups, BJ = BJ1035 samples and Bov = uninfected bovine lymphoblast samples. Each assay was carried out in triplicate. Any group with a product observed was cloned and subsequently sequenced.

3.4 Specific cDNA method

To confirm whether or not the remaining fifteen candidates were real miRNAs or not a second PCR approach was used. This method was adapted from (Varkonyi-Gasic *et al.*, 2007) but did not include a SYBR Green assay and was carried out as described in sections 2.2.4 and 2.2.7. The stem-loop RT primer covers the last six nucleotides of the miRNA and the forward primer covers the full length of the miRNA excluding the last six nucleotides. Therefore sequencing of the resulting product would be meaningless as any sequence would contain only the primer sequences. For this reason a product observed by agarose gel electrophoresis in the BJ1035 sample but not bovine lymphoblast sample was taken as a positive result. Five out of fifteen putative miRNAs were confirmed by this method (Figure 3.3 A). Minus RT controls for BJ1035 samples positive for the miRNA of interest were performed to ensure amplification was not from viral genomic DNA (Figure 3.3 B).

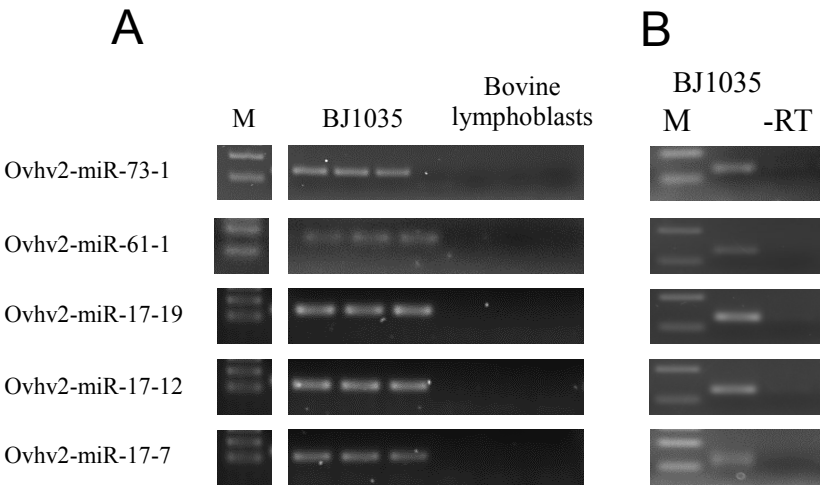


Figure 3.3. Analysis of OvHV-2-encoded miRNA expression using miRNA specific RT-PCR.

A) Expression of five predicted OvHV-2-encoded miRNAs in BJ1035 cells but not uninfected bovine lymphoblasts was confirmed by miRNA specific RT-PCR. Each assay was carried out in triplicate. M : 50bp and 100bp markers. B) miRNA specific RT-PCR was carried out along with a no RT (-RT) control.

3.5 Discussion

A total of twenty-seven miRNAs were confirmed by either PCR method bringing the total number of confirmed miRNAs to thirty-five. Table 3.1 lists the miRNAs confirmed with their consensus sequence, 5' and 3' co-ordinates in the OvHV-2 genome (accession no. AY839756.1) and validation method. All the miRNAs validated are on the minus strand i.e. they are transcribed in the same orientation, right to left. Figure 3.4 shows the OvHV-2 genome with relative locations of confirmed miRNAs. By convention, virus-encoded miRNAs are named in relation to the nearest ORF transcribed in the same direction as the miRNA. Confirmed miRNAs have been named according to this nomenclature, including those confirmed by Dr Levy, previously designated ovhv2-miR-1 to 8. Two miRNAs are encoded at the left hand end of the genome between the terminal repeat region and 3' end of ORF Ov2 and are named ovhv2-miR-Ov2-1 and -2. Like AlHV-1 and equine herpesvirus 2 (EHV-2), OvHV-2 also contains two large regions of the genome where no ORFs are predicted (Hart *et al.*, 2007). The majority of the miRNAs (thirty out of thirty-five) are located in two clusters in the larger of these two regions. Twenty seven miRNAs are located at the left hand of this region in a cluster spanning 4377 bp and a smaller cluster containing three miRNAs are located at the right hand of this region spanning 320 bp. These miRNAs have been named ovhv2-miR-17-1 to -30. One miRNA is encoded in a non-coding region towards the right end of the genome and is designated ovhv2-miR-73-1. Finally, two miRNAs are encoded within ORFs 24 and 61 and are consequently named ovhv2-miR-24-1 and ovhv2-miR-61-1 respectively. This work was published in 2014 and the paper can be found in Appendix 11 (Nightingale *et al.*, 2014).

The number of miRNAs expressed by individual herpesviruses ranges from at least three to fifty (Kincaid & Sullivan, 2012) and OvHV-2 encodes at least thirty-five miRNAs. An algorithm used by (Walz *et al.*, 2010) predicted sixty-one hairpin sites in the OvHV-2 genome that might encode miRNAs. Of these, 32 have been confirmed to be expressed. Three of the miRNAs (ovhv2-miR-24-1, -61-1 and -73-1) confirmed by the PCR methods used here were not predicted by Walz *et al.*. CLASH was performed by a previous PhD student and will be described in further detail in section 4.1 (Riaz, 2014). Although the main aim of this method is to elucidate targets of miRNAs the data can also be used as a validation of the first RNA sequencing assay performed by Dr Levy. Thirty-three of the thirty-five miRNAs confirmed by either PCR or northern hybridisation were found in the CLASH data. ovhv2-miR-24-1 and -61-1 were the two miRNAs not detected in the CLASH protocol; these miRNAs were also the only two miRNAs confirmed by PCR that only had one read in the small RNA sequencing data. None of the miRNAs, not confirmed by PCR but predicted by Dr Levy, were detected in

the CLASH protocol providing further support that the miRNAs confirmed by PCR are expressed by the virus.

The lack of a tissue culture system for OvHV-2 means that investigating miRNAs in the context of infection is challenging, unless performed in the rabbit model of MCF which is out with the scope of this project. The small RNA sequencing originally performed by Dr Levy is a snapshot of what is happening at that precise moment in the cell; expression of virus-encoded miRNAs is often highly context dependent (Forte & Luftig, 2011) and the miRNAs not detected by PCR may be subject to tight regulation of their expression. The BJ1035 cells do not represent a natural infection of OvHV-2 as the virus does not appear to be truly latent but also does not go through a full replication cycle resulting in the production of virus. It is therefore entirely possible that the ten miRNAs not found are only expressed in the context of a natural infection and would therefore not be detected by any of the methods used in BJ1035 cells.

One of the reasons PCR was chosen over northern hybridisation for validating the remaining candidate miRNAs was the potential for quantification, however this was not performed. As the only available material to work with within the scope of the project was the BJ1035 cells it was deemed that quantification would not necessarily add any insight into virus biology. miRNA* candidates were also not investigated however there is potential to use the PCR methods described to carry out validation of these in the future if it becomes of interest. Mapping the transcripts and promoter region of the large cluster of OvHV-2-encoded miRNAs by northern hybridisation was unsuccessfully attempted by Dr Riaz. Mapping of promoter regions for other OvHV-2-encoded miRNAs was not attempted as it was out with the scope of this project and characterisation of the targets of OvHV-2-encoded miRNAs was deemed to be more important than the characterisation of their expression.

Table 3.1 OvHV-2-encoded miRNAs

ovhv2-miR-	Previous name	Abundance RNA-seq	Walz *et al.*	5'nt	3'nt	Sequence	Validation Method
Ov2-2	miR-1	10588	√	927	906	AAGGCUUGAUAAGUAGCACUGA	Levy *et al.*
Ov2-1		253	√	1182	1161	AUGCUUGUUUAGGCCCCAUGAA	Sequencing
17-30		434	√	27722	27702	UUUGGGUGUCUCCUGUCAUCU	Sequencing
17-29	miR-2	39169	√	27903	27881	AUCUUGGACGCAUCUGUCAGUAG	Levy *et al.*
17-28		6200	√	28066	28045	UCUAGGUUGCAUUUUGCUGUAG	Sequencing
17-27		6015	√	28209	28188	CCCACAUUUAAGGUGCUCGUGU	Sequencing
17-26		322	√	28381	28361	AUAUUCGUUUAGACGCAAGUA	Sequencing
17-25		4717	√	28515	28495	CAAUGCUGCUUUGGUGCCUCA	Sequencing
17-24		1014	√	28683	28661	GGGUUCCUCGAGUGGAUAUUGUU	Sequencing
17-23		21686	√	28781	28761	AUACACACUGAAAGAGCUAGA	Sequencing
17-22		740	√	28888	28866	AUAAGGCCAACACUAGGUGCUGU	Sequencing
17-21		31227	√	29052	29028	AAGCACCUUGGGUGAUGUCUCUGUU	Sequencing
17-20	miR-3	14694	√	29248	29226	UCUGUAUCAUAGGGGUUGUGUUG	Levy *et al.*
17-19		6487	√	29345	29323	AAGCAUAGCUGGGAGUGUCUAGA	Specific cDNA
17-18		9219	√	29455	29434	UAGUAGUCCGUUAACGCAAAGU	Sequencing
17-17	miR-4	17508	√	29637	29616	AAGGAUCCUUAAGUGACGAACG	Levy *et al.*
17-16		8095	√	29745	29725	UAAACUGGUGGUAGGCGGUCU	Sequencing
17-15		2967	√	29945	29923	UAGCAGUUAUGCAGGUAUCUGGU	Sequencing
17-14		3794	√	30091	30067	UGGCAUUUCCAGGAGCCUGUUGUUC	Sequencing
17-13		24497	√	30338	30316	UUGGGUCCAACAUGAGACGCGGU	Sequencing
17-12		14966	√	30510	30489	UAUGUCAGAAGUGAAGCUGAGA	Specific cDNA

17-11		1357	√	30632	30611	UGGUUUGCAUCUGCACCCAGUU	Sequencing
17-10	miR-5	11187	√	30818	30796	UGAAGUUACAGCUGCACCUGGAU	Levy *et al.*
17-9		5457	√	30980	30956	UAGAGUUACUAAGGAUUCCCUGGUA	Sequencing
17-8		942	√	31085	31066	AAUCGCCGGUGGCCUUCUAG	Sequencing
17-7		351	√	31359	31340	UAUAGACGGGUAUGCUGCCG	Specific cDNA
17-6	miR-6	10378	√	31462	31440	UAUUUUUAGCGGAGACCUCUAGG	Levy *et al.*
17-5		1535	√	31603	31582	CCUUUUUGGUGAGUUGCCCUGU	Sequencing
17-4		517	√	32075	32054	GAUUUGAUAAAGCCUGCCUGCG	Sequencing
17-3	miR-7	16574	√	36313	36289	GAAGGCGCAUCAUAGACACCACUUC	Levy *et al.*
17-2		7834	√	36462	36441	ACCCCGGGGGUAUGUGCAGGAC	Sequencing
17-1	miR-8	46048	√	36575	36554	UGGCUCAGCGUGACUGCUCUUC	Levy *et al.*
24-1		1	x	48585	48560	GAGCAGUACUACACAGCAGACAACAU	Sequencing
61-1		1	x	96435	96411	UUGGGGACGUGCUGGCUGACGACGU	Specific cDNA
73-1		6064	x	117122	117100	UAAUCUCUGCUCCAAUUGUAAAU	Specific cDNA

The 35 validated miRNAs are listed. **Previous name**: designation in Levy *et al.* 2012. **Abundance (RNA-seq)**: Total number of sequence tags representing the miRNA in the RNA-seq data. **Walz *et al.***: miRNAs predicted by Walz *et al*. √ = yes; X= no. **5'nt/3'nt**: the first and last nucleotides of the mature miRNA on the OvHV-2 genome (AY839756). **Sequence**: sequence of the mature miRNA. **Validation method**: Levy *et al*.: validated previously; Sequencing: validated using RT-PCR and subsequent sequencing; Specific cDNA: validated using the specific cDNA method.

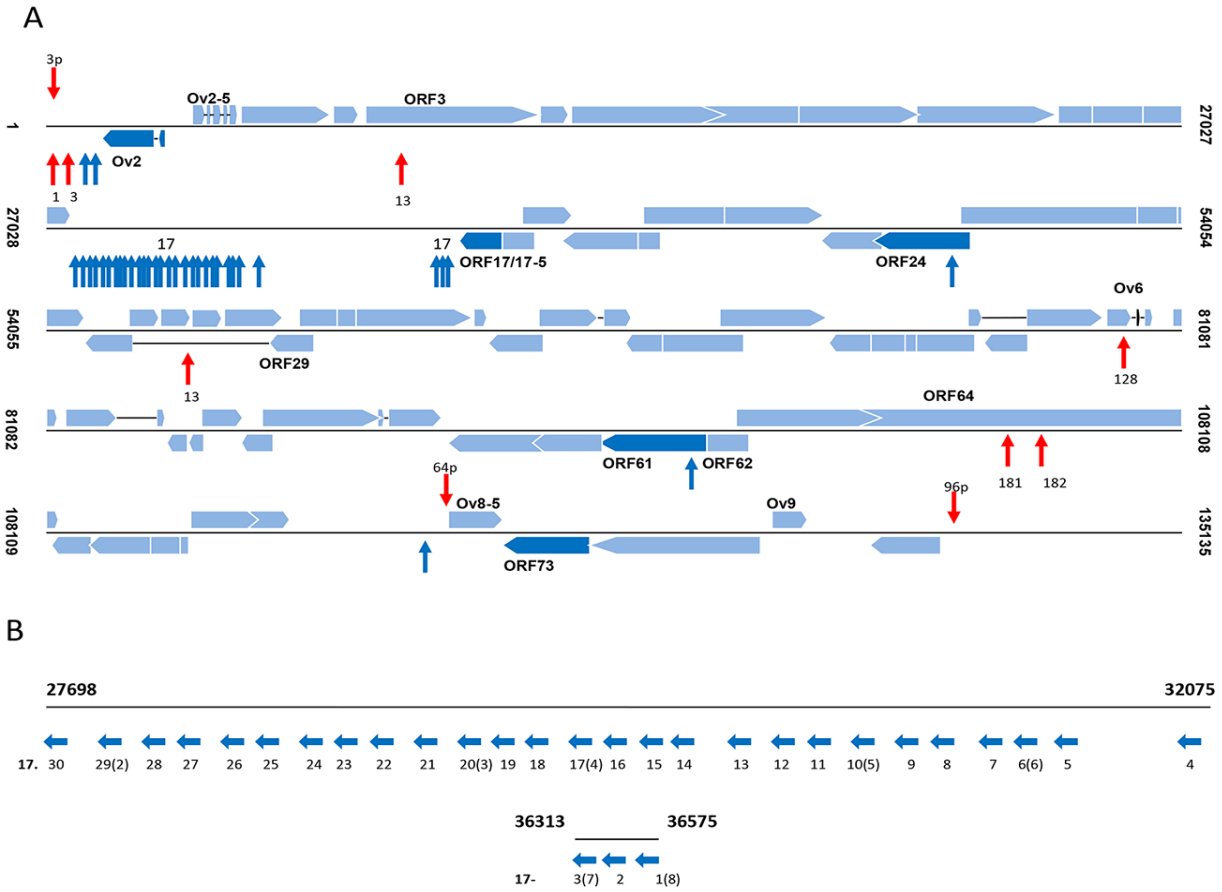


Figure 3.4. Location of miRNAs in the OvHV-2 genome.

A) The relative positions of the predicted miRNAs in the OvHV-2 genome are shown. Numbering is from Hart *et al*. The genome is represented by a thin line and ORFs are indicated by blue boxes. Boxes above the line represent ORFs transcribed left to right; those below the line represent ORFs which are transcribed right to left. Only those ORFs adjacent to predicted miRNAs are named. Arrows indicate the position of the predicted miRNAs; arrows above the line represent miRNAs transcribed left to right, those below the line represent miRNAs which are transcribed right to left. Dark blue vertical arrows indicate validated miRNAs, Red arrows indicate non-validated miRNAs named according to their groups (see Figure 3.2). Those OvHV-2 ORFs closest to validated miRNAs and after which those miRNAs are named are shown in dark blue. B) The locations of ovhv2-miR-17-1 to -30 are shown in more detail. Numbers in brackets are the previous names of miRNAs validated by C. Levy.

Chapter 4: Identification and Validation of cellular targets of OvHV-2-encoded miRNAs

4.1 Introduction

Identification of cellular targets of OvHV-2 miRNAs is necessary in order to support the hypothesis that virus-encoded miRNAs may play a role in the differences observed in pathogenesis of MCF and to begin to explain the difference between the outcome of OvHV-2 infection in sheep and cattle. The effect of virus-encoded miRNAs on pathogenesis is well documented in a number of herpesviruses including MDV, KSHV and EBV. MDV encodes a miR-155 homologue, mdv1-miR-M4, shown to be critical in the development of lymphomas. Upon deletion of this miRNA the ability of the virus to induce lymphomas was abolished (Zhao *et al.*, 2011), however this has not been confirmed in very virulent strains of MDV (Yu *et al.*, 2014). KSHV also encodes a miR-155 homologue which may also contribute to the induction of B cell tumours (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007). Although EBV does not encode a miR-155 homologue it has been shown to induce cellular miR-155 expression (Linnstaedt *et al.*, 2010) allowing for B cell immortalisation. It has also been shown that EBV-encoded miRNAs play a role in cellular transformation; recombinant EBV that lacks the BHRF1 cluster of miRNAs displays reduced transforming capability (Feederle *et al.*, 2011). The development of MCF in susceptible species is poorly understood and it is possible that OvHV-2-encoded miRNAs play a role. It is likely that OvHV-2 co-evolved with sheep, its natural host, and therefore it is likely that virus-encoded miRNAs also evolved to target sheep genes. When OvHV-2 infects a susceptible host virus-encoded miRNAs may not target the same pathways as in sheep or may target entirely different pathways which may contribute to MCF pathogenesis. It is not only important to elucidate targets of OvHV-2-encoded miRNAs, but also determine whether there is differential targeting of sheep and cattle transcripts, and whether this could explain any differences observed in the pathogenesis of the virus between the two closely related species.

Previous work looking a target identification used a biochemical technique called CLASH (described in section 1.6.4). SEFs were transduced with a lentivirus containing ovhv2-miR-17-1, -2 and -3 and CLASH was performed (Riaz, 2014). Delta-like 1 (*DLL1*), a ligand for Notch receptor signalling, was the only gene to be identified in all 3 biological replicates and as a differentially enriched gene this was taken forward for validation.

Another method for target identification is to determine whether any OvHV-2-encoded miRNAs share homology to any cellular miRNAs; and it was shown (Levy, 2011) that ovhv2-miR-73-1 is homologous to miR-216a (Figure 3.1). miR-216a has been shown to target phosphatase and tensin homologue (*PTEN*), a negative regulator of Akt signalling, and Y box

binding protein 1 (*YB-1*), a DNA binding protein (Kato *et al.*, 2009; Kato *et al.*, 2010). Both *PTEN* and *YB-1* were chosen for validation as targets of ovhv2-miR-73-1.

A final method for target identification is to use bioinformatics. For the purposes of this thesis, RNAHybrid was chosen as the preferred prediction programme. Based on the fact that many viruses interact with the MHC class II pathway as a mechanism of immune evasion it was hypothesised that MHC class II genes could be a target of OvHV-2-encoded miRNAs (Zuo & Rowe, 2012a).

4.2 Aims

The aim of this part of the project was to identify cellular targets of OvHV-2-encoded miRNAs using a combination of biochemical and bioinformatic prediction methods. Differences in targeting between sheep and cattle were also investigated to determine whether miRNAs play a role in the differences observed in pathogenesis of MCF between the two closely related species.

4.3 Validation of DLL1 as a target of OvHV-2-encoded miRNAs

4.3.1 DLL1 introduction

DLL1 is one of the ligands for the Notch receptor and binding of DLL1 to Notch causes activation of the Notch signalling pathway. DLL1 is a membrane-bound ligand and as such activation of Notch signalling is cell-cell mediated. Notch was originally identified as a pleiotropic regulator of cell fate, in almost every cell type, and as a major determinant in lymphocyte differentiation (Andersson *et al.*, 2011; Guruharsha *et al.*, 2012). Since the initial discovery of Notch, subsequent research has expanded our understanding of the roles of Notch further into control of cell lineages in the innate immune system, as well as regulation of T cell function and differentiation. Inactivation of Notch signalling leads to a block early in T cell development (Radtke *et al.*, 2013). It was observed *in vitro* that DLL1 could completely block B cell differentiation while promoting emergence of cells with a T cell/natural killer cell precursor phenotype (Jaleco *et al.*, 2001). As OvHV-2 infects T cells, the ability of the virus to modulate T cell function is likely to be key to a successful latent infection. It has been shown that EBV infects any B cell and drives it into a memory B cell phenotype enabling a circulating population of infected cells to persist (Thorley-Lawson, 1998). OvHV-2 may also need to alter the phenotype of infected T cells to allow for persistence. It therefore makes sense that Notch signalling may be altered and down-regulation of *DLL1* leading to lower levels of Notch signalling could be favourable for maintenance of latency of OvHV-2 in sheep. If the targeting of *DLL1* in sheep can be confirmed and there is differential targeting of *DLL1* in cattle (i.e. no or little targeting of *DLL1*) this could be a contributing factor promoting latency in sheep in contrast to the progression of infected cattle cells into large granular lymphocytes characteristic of MCF.

Canonical Notch signalling begins with binding of one of five ligands belonging to either the Jagged family (Jagged 1 and Jagged 2) or Delta-like family (DLL1, DLL3 and DLL4) to the Notch receptor. This triggers two proteolytic cleavage events which release the Notch intracellular domain (NICD) from the membrane. NICD then translocates to the nucleus where it binds the J kappa-recombination signal binding protein (RBPJ) family of transcription factors inducing expression of downstream genes (Radtke *et al.*, 2013). Published Notch target genes include transcription factors belonging to the hairy/enhance of split family (these genes play a key role in development and cell fate decision), the transcription factor *GATA3* which is a master regulator of T cell development and Th1/Th2 lineage commitment, and interleukin 2 receptor, alpha chain (*IL2RA* or *CD25*) (Borggrefe & Oswald, 2009).

Interaction with RBPJ by both EBV and KSHV is well-documented. It has been shown that EBNA2, the major initiator of transcription of genes involved in type III latency, and NICD have partially interchangeable functions in regards to B cell target gene expression (Rowe *et al.*, 2014; Zimber-Strobl & Strobl, 2001). In KSHV, ORF50, the major reactivation protein of the virus, interacts with RBPJ (Liang *et al.*, 2002b). It is interesting that KSHV uses constitutive activation of Notch signalling as a strategy for reactivation from latency whereas EBV uses the Notch pathway to enter latency. If OvHV-2 modulates Notch signalling it is not clear which aspect of virus biology it would influence.

Although no virus-encoded miRNAs have been shown to target *DLL1* or the Notch signalling pathway, the cellular miRNA miR-1 has been shown to negatively regulate DLL1 protein levels in mouse embryonic stem cells (Wang *et al.*, 2010).

DLL1 was identified as a target of ovhv2-miR-17-2 in CLASH performed by Dr Riaz (Riaz, 2014). The role DLL1 and Notch signalling may play in MCF pathogenesis, and the fact that *DLL1* formed the most hybrids in all three CLASH tests made it a prime candidate for further investigation. Initial validation of *DLL1* as a target of ovhv2-miR-17-2 and luciferase assays showed an approximately 60% down-regulation of relative luciferase levels with ovhv2-miR-17-2 compared to a scrambled siRNA with a luciferase construct containing two *DLL1* target sites (Riaz, 2014). Due to time constraints, mutagenesis of the target sites was not performed. Analysis of other *DLL1* target sites revealed by CLASH, and examination of the cattle *DLL1* sequences for target sites was also not performed.

4.3.2 RNAHybrid analysis of *DLL1* mRNA sequences

RNAHybrid was used to investigate the predicted sheep *DLL1* mRNA sequence (accession number XM_004011569.1) and the cattle *DLL1* transcript variant 1 mRNA sequence (XM_002690401.2) for targets of ovhv2-miR-17-2. Analyses can be seen in Figure 4.1. Targets identified by RNAHybrid had perfect complementarity between nucleotides 2 and 8 of the miRNA and no G:U base pairing in that region.

Analysis of the sheep *DLL1* mRNA sequence revealed three potential binding sites of ovhv2-miR-17-2, located in the coding region. The site starting at position 2642 (site 1) was the primary target site identified in CLASH and was used in the initial luciferase assays (Riaz 2014). The other two sites revealed by RNAHybrid were also found as hybrids in the CLASH data. One of these, starting at position 2591 (site 3) is in close proximity to site 1. The other site, starting at position 1241, was not included in the validation work described in subsequent sections. A fourth target site, not shown by RNAHybrid in this analysis was also identified in CLASH; nucleotides 2644-2650 are the nucleotides bound by the seed site of ovhv2-miR-17-

2 (site 2). This site overlaps site 1 which is why it was not identified by RNAHybrid and was therefore also included in the initial luciferase assay.

Analysis of the cattle *DLL1* coding region only gave rise to one predicted binding site for ovhv2-miR-17-2, the position of which was homologous to site 3 in the sheep *DLL1* sequence. Sites 1 and 2 were not present in the cattle *DLL1* mRNA sequence. Analysis of the cattle *DLL1* mRNA sequence also identified two ovhv2-miR-17-2 binding sites in the 5'UTR region (RNAHybrid analysis not shown).

It should be noted that the sequence of sheep *DLL1* in Ensembl (ENSOART00000005568) differs to the sequence used here for RNAHybrid analysis and that NCBI now has an updated sequence for *DLL1* (XM_012183355.1). The differences observed between sequences will be discussed further in section 4.3.4.

```
TARGET : Sheep_DLL1
length: 3021
MIRNA  : ovhv2-miR-17-2
length: 22

mfe: -39.9 kcal/mol
p-value: undefined

position  1241
target 5' U      C  A          A 3'
           UCCUGC CG GCUCCCGGGG
           AGGACG GU UGGGGGCCCC
miRNA  3' C      U  A          A 5'

TARGET : Sheep_DLL1
length: 3021
MIRNA  : ovhv2-miR-17-2
length: 22

mfe: -32.9 kcal/mol
p-value: undefined

position  2591 (site 3)
target 5'  A       GA    G       C 3'
            CCUGUGC  GUGU CCCGGGG
            GGACGUG  UAUG GGGCCCC
miRNA  3' CA             G       A 5'

TARGET : Sheep_DLL1
length: 3021
MIRNA  : ovhv2-miR-17-2
length: 22

mfe: -30.6 kcal/mol
p-value: undefined

position  2642 (site 1)
target 5' U   CGGGGAGG    CCCG  A       G 3'
           UCC         GCGC    AC CCCGGGG
           AGG         CGUG    UG GGGCCCC
miRNA  3' C   A           UA    G       A 5'

TARGET : Cattle_DLL1
length: 5060
MIRNA  : ovhv2-miR-17-2
length: 22

mfe: -34.8 kcal/mol
p-value: undefined

position  2381
target 5'  A     GUGA    G       C 3'
            CCUGU    GUGC CCCGGGG
            GGACG    UAUG GGGCCCC
miRNA  3' CA     UG      G       A 5'
```

Figure 4.1 RNA Hybrid analyses of Sheep and Cattle DLL1 mRNA sequences for target sites of ovhv2-miR-17-2

Mfe = minimum free energy. Constraints on RNAHybrid analysis included perfect complementarity between nucleotides 2 and 8 with no G:U pairing. Positions refer to nucleotide start sites in accession numbers referenced in section 4.3.2. Targets shown are located in the coding region.

4.3.3 Validation of *DLL1* as a target of ovhv2-miR-17-2 by luciferase assay and mutagenesis of target sites

Due to the changes in annotation of the *DLL1* mRNA sequence since the initial luciferase assays (Riaz 2014), and the fact that site 3 was in close proximity to sites 1 and 2, new luciferase constructs were made to include site 3. Oligonucleotides containing nucleotides 2580-2682 were cloned into psiCHECK-2. Three mutated oligonucleotides were designed; one with site 1 mutated into a *Bam*HI restriction enzyme site, one with site 3 mutated into a *Bam*H1 restriction enzyme site and one with both site 1 and site 3 mutated into *Bam*HI and *Eco*RV restriction enzyme sites respectively. Restriction enzyme sites were chosen to substitute seed sequence sites as they ablated ovhv2-miR-17-2 binding and served as a diagnostic tool for cloning. Site 2 was not mutated independently as mutation of this site would disrupt the 3' of ovhv2-miR-17-2 binding to site 1.

Luciferase assays were carried out as described in section 2.8.1. psiCHECK-2 expresses both *Renilla* luciferase and firefly luciferase. The multiple cloning site is located in the 3'UTR of *Renilla* luciferase and firefly luciferase acts an internal control. *Renilla* luciferase expression is normalised to firefly luciferase expression so that any changes in *Renilla* luciferase expression are due to co-transfection of miRNA mimics. Figure 4.2 shows the combined results of two independent luciferase assays with n = 12. No significant knockdown was observed when an ovhv2-miR-17-2 mimic was co-transfected with psiCHECK-2 compared to a scrambled siRNA control. When the same mimic was co-transfected into cells with a psiCHECK-2 construct containing the *DLL1* sequence described above a 35% reduction in relative luciferase expression was observed compared to a scrambled siRNA control ($p \leq 0.0001$). When an ovhv2-miR-17-2 mimic was co-transfected with psiCHECK-2 constructs containing site 1 mutated or site 3 mutated *DLL1* constructs approximately 30% ($p \leq 0.01$) and 25% ($p \leq 0.0001$) reductions in relative luciferase expression was observed compared to a scrambled siRNA control. When an ovhv2-miR-17-2 mimic was co-transfected with a psiCHECK-2 construct containing both site 1 and site 3 mutations in the *DLL1* sequence relative luciferase levels were restored with no significant difference observed between scrambled siRNA and ovhv2-miR17-2.

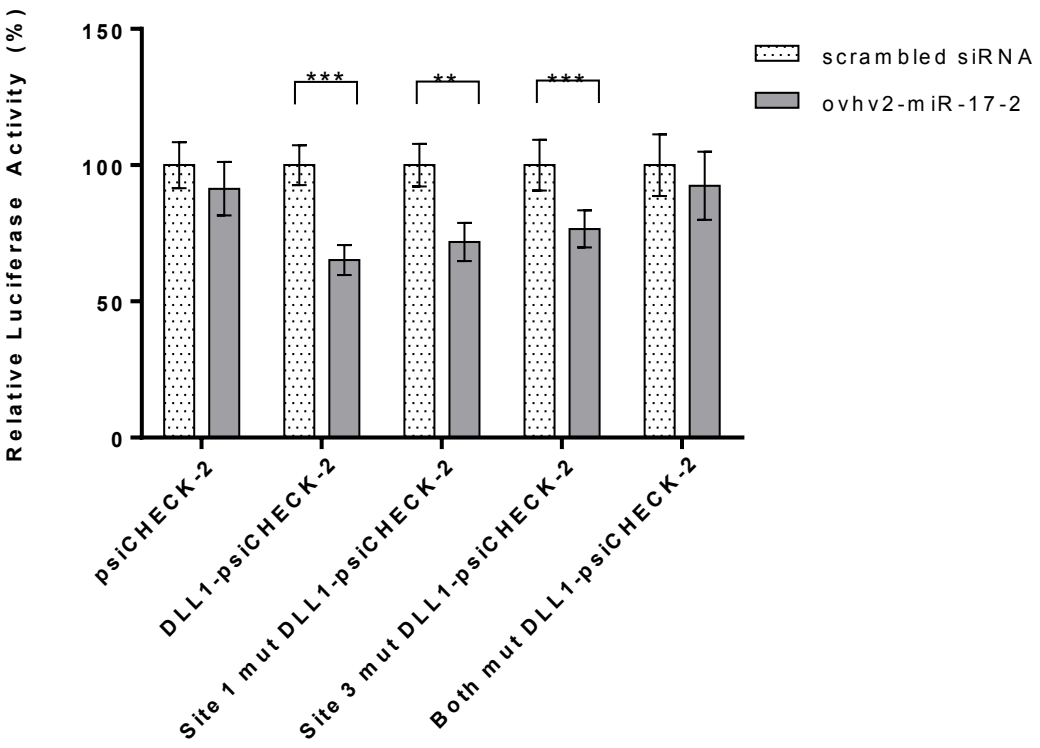


Figure 4.2 Relative luciferase expression levels of DLL1-psiCHECK-2 and mutant DLL1-psiCHECK-2 constructs with ovhv2-miR-17-2 compared to a scrambled siRNA

293T cells were co-transfected with a psiCHECK-2 construct containing a 100 bp region of DLL1 containing the predicted miRNA sites; or with the first site mutated, the third site mutated or both the first and third site mutated and an ovhv2-miR-17-2 mimic or a scrambled siRNA control. After 48 hrs, *Renilla* luciferase levels were measured, normalised to firefly luciferase levels and expression in control and test miRNA compared. ** = $p < 0.01$, *** = $p < 0.0001$

4.3.4 Analysis of genome sequences of sheep *DLL1*

Comparison of the sheep *DLL1* mRNA sequence in NCBI (XM_004011569.1) and Ensembl (ENSOART00000005568) revealed striking differences in the coding region of *DLL1*, which is summarised in Figure 4.3 (Appendix 3 for alignments). At the genomic level, the sequences in NCBI and Ensembl are identical. The NCBI *DLL1* mRNA sequence is 3021 bp long whereas the Ensembl *DLL1* mRNA sequence is only 1845 bp long. The main difference between these two sequences is that the 5' end of the NCBI sequence is not present in the Ensembl sequence, which does not start with an ATG codon. This implies that the start site of the coding region of *DLL1* may be incorrectly annotated in Ensembl. The other main region of variation between the two sequences is located towards the 3' of the sequence, where three of the predicted ovhv2-miR-17-2 binding sites are located. In Ensembl, the distance of site 3 from site 1 is approximately 200 bp more than in NCBI. Site 3 is located in a different exon to sites 1 and 2; the intron in between is largely unannotated hence the precise intron/exon boundary cannot be fully mapped.

4.3.5 Amplification of the *DLL1* region containing ovhv2-miR-17-2 target sites

In order to determine whether NCBI or Ensembl had the correct sequence in regards to the location of ovhv2-miR-17-2 target sites, primers were designed to amplify the region spanning the three sites (Appendix 2 for sequences), from nucleotides 2416 to 2745 of the NCBI sheep *DLL1* sequence (Figure 4.3 "c"). PCR was performed on cDNA made from sheep mesenteric lymph node RNA (a kind gift from Louise Nicol). Sequencing of the PCR product revealed that the only ovhv2-miR-17-2 binding site present was site 3, and that the sequence obtained was highly similar to the bovine *DLL1* sequence. To elucidate the intron/exon boundary site that was mapped differently in NCBI and Ensembl versions of *DLL1*, the same primers were used to amplify the region from SEF genomic DNA. The PCR product from the genomic DNA was identical in size and sequence to that obtained from cDNA showing that there is no intron in this region.

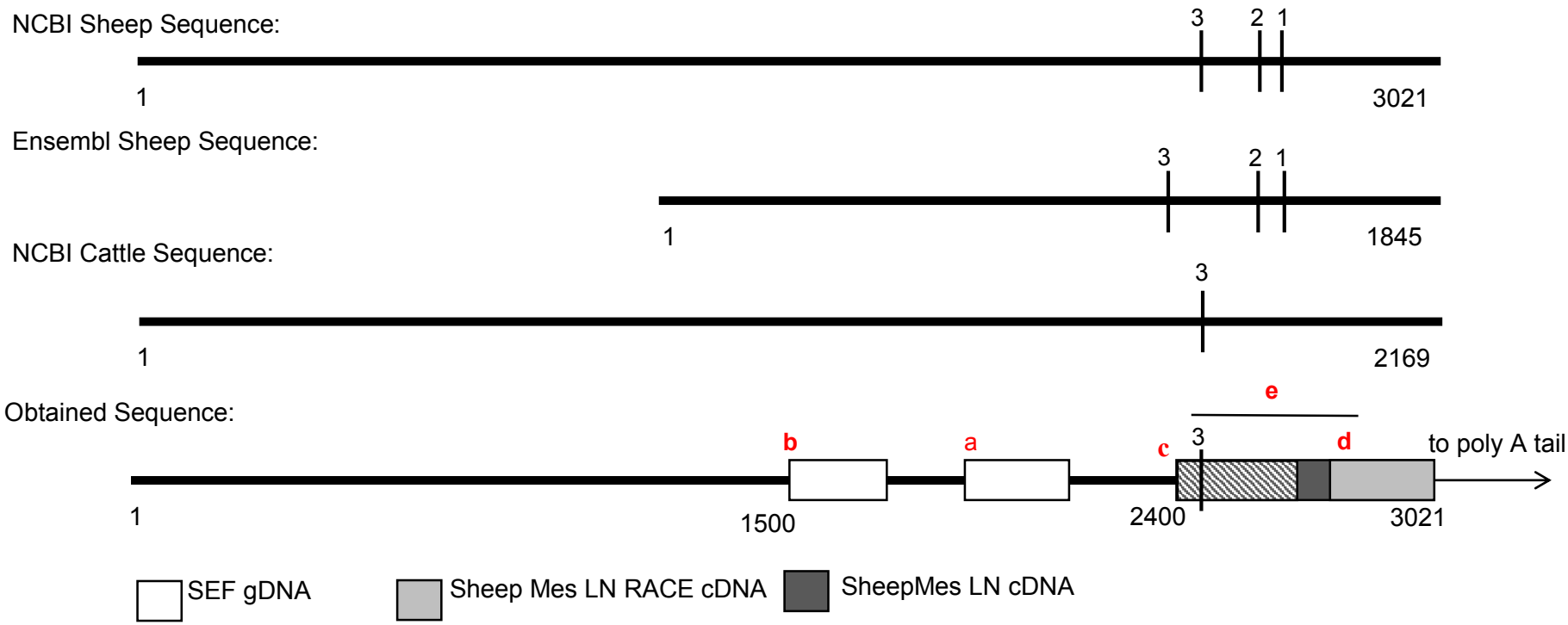


Figure 4.3 Schematic of aligned DLL1 sequences

Black horizontal bars represent coding sequence, with numbers representing nucleotide number. Black vertical bars represent target binding sites for ovhv2-miR-17-2. White boxes represent obtained sequence of DLL1 from SEF gDNA. The light grey box represents sequence obtained from 3' RACE PCR performed with mesenteric lymph node cDNA. The dark grey box represents sequence obtained from mesenteric lymph node cDNA. The diagonal striped dark grey and white box represents a region where both SEF gDNA and mesenteric lymph node cDNA have been used to obtain sequence. Small letters a to e represent the order in which sequencing was performed.

4.3.6 Validation of confirmed *DLL1* sequence as a target of ovhv2-miR-17-2 by luciferase assay

Primers tagged with appropriate restriction enzymes (forward primer with *Xho*I and reverse primer with *Not*I, Appendix 2) were used to amplify nucleotides 2416-2745 and were subsequently cloned into psiCHECK-2. Luciferase assays were carried out as described in section 2.8.1 with n = 12 for each plasmid. A statistically significant (p = 0.001) reduction of 25% of relative luciferase expression levels was observed when ovhv2-miR-17-2 was co-transfected with the psiCHECK-2 plasmid containing *DLL1* sequence compared to the scrambled siRNA (Figure 4.4). No significant knockdown was observed when an ovhv2-miR-17-2 mimic was co-transfected with psiCHECK-2 compared to a scrambled siRNA control.

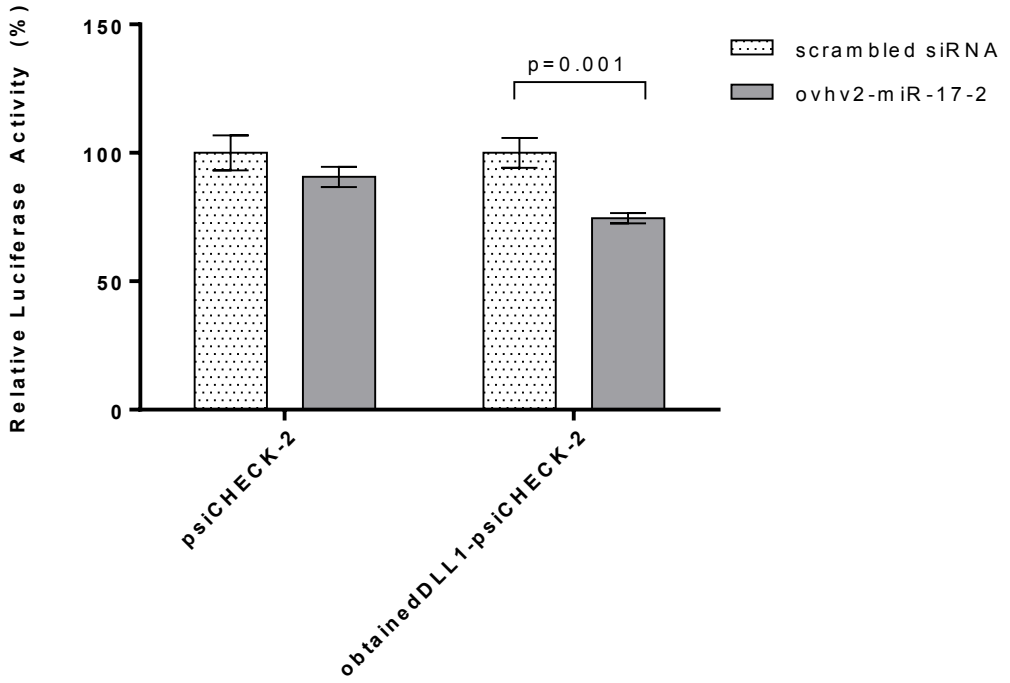


Figure 4.4 Relative luciferase expression levels of obtainedDLL1-psiCHECK-2 with ovhv2-miR-17-2 compared to a scramble siRNA

293T cells were co-transfected with a psiCHECK-2 construct containing a 330 bp region of DLL1 (diagonal striped dark grey and white box in Figureure 4.3), and an ovhv2-miR-17-2 mimic or a scrambled siRNA control. After 48 hrs, *Renilla* luciferase levels were measured, normalised to firefly luciferase levels and expression in control and test miRNA compared.

4.3.7 Amplification of *DLL1* and RACE PCR

Two regions of the *DLL1* gene were chosen for amplification in order to develop an RT-qPCR assay (Figure 4.3 "a" and "b"). Primers were designed to amplify a 150 bp region (nucleotides 1970 to 2119, Figure 4.3 "a"). PCR was performed on SEF gDNA and subsequent sequencing revealed a 130 bp insert in the middle of the sequence of the PCR product which partially aligned to the genomic sequence of *DLL1* (Appendix 3). Region "b" was designed to amplify a 218 bp region from nucleotide 1538-1756. PCR was performed on SEF gDNA and the sequence obtained matched the NCBI sheep *DLL1* sequence. Due to difficulty in finding a sheep cell line that expressed DLL1 and the inconsistencies in the reported sequences of *DLL1* the RT-qPCR was not developed any further.

To determine whether the ovhv2-miR-17-2 binding sites 1 and 2 were present in the cDNA of *DLL1*, 5' and 3' RACE PCR were performed. Primers were designed using the sequences obtained from the hybrids in CLASH (Appendix 2 for primers). RACE PCR was performed on cDNA made from sheep mesenteric lymph node. The PCR product was analysed by agarose gel electrophoresis and one band was extracted and purified from the 3' RACE sample. The remaining 3' RACE PCR product and 5' RACE PCR product were purified and cloned into pCR2.1-TOPO. A number of colonies were picked and a restriction digest performed. Four 5' RACE colonies were sent for sequencing, however none contained the primer sequence or mapped to *DLL1*, and this was not investigated further. Of the seven colonies sent for sequencing from the 3' RACE PCR purified product, one was 99% identical to goat (XM_005685019.1) and 97 % identical to cattle (XM_002690401.2) *DLL1* (Appendix 3). The seven colonies from the 3' RACE extracted band also all mapped to goat and cattle *DLL1* with partial alignment to the sheep genome (Appendix 3).

The sequence obtained from 3' RACE did not contain ovhv2-miR-binding site 1 or site 2, and did not overlap with the sequencing obtained in section 4.3.5. Primers were designed to amplify the region between these two sites (Figure 4.3 "e", Appendix 2 for primer sequence). PCR was performed on sheep mesenteric lymph node cDNA and the sequence obtained again aligned to the cattle *DLL1* sequence. A combined sequence was made from the sequence obtained in section 4.3.5, the 3' RACE sequencing and the sequence joining those two regions (Appendix 3). This sequence had no significant similarity to the sheep *DLL1* cDNA sequence in NCBI but did partially map to chromosome 8 in the sheep genome assembly OARv3.1, in the region where *DLL1* is predicted (Appendix 3). Importantly, the ovhv2-miR-17-2 binding sites 1 and 2 were not present in this sequence, and as such this work was not continued.

4.4 Investigating targets of ovhv2-miR-73-1 based on its homology to miR-216a

4.4.1 PTEN Introduction

PTEN is a well-known tumour suppressor (Song *et al.*, 2012). Its primary role is to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3), preventing accumulation of PIP3 in the cell allowing Akt (v-Akt murine thymoma viral oncogene homolog 1) activation and downstream signalling (Maehama & Dixon, 1998; Stambolic *et al.*, 1998). PIP3 is generated by the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to PIP3 by phosphatidylinositol-3-kinase (PI3K) which occurs when the cell receives growth stimulation signals (Salmena *et al.*, 2008). Akt is recruited to the site of PIP3 by direct interaction with its pleckstrin homology domain; the action of two kinases subsequently activate Akt. Akt can be considered a hub protein as it is able to interact with a large number of proteins in the cell. The outcomes of these interactions can be loosely classed into a few major pathways; survival, proliferation and cell growth (Vivanco & Sawyers, 2002). Akt also plays a role in metabolism, angiogenesis and glucose uptake, which are of high significance in enabling tumorigenesis (Manning & Cantley, 2007). Figure 4.5 summarises some of the downstream targets of Akt signalling and the pathways that are affected.

Given that an OvHV-2 infected cell needs to be able to avoid apoptosis, induce cell growth, and increase glucose uptake and metabolism to cope with virus replication; and that uncontrolled proliferation is one of the hallmarks of MCF pathogenesis it was hypothesised that targeting of the Akt pathway by OvHV-2 would be consistent with the observed biology. The suppression of apoptosis is of particular importance for herpesviruses which need to maintain a latent state. In EBV, LMP1 can activate PI3K, stimulating Akt activation, which significantly contributes to B cell transformation (Chen, 2012). It has been shown that Akt signalling may also be required for reactivation of EBV from latency (Iwakiri & Takada, 2004). EBV also encodes a miRNA, miR-BART7-3p that specifically targets *PTEN* and has been shown to promote epithelial-mesenchymal transition and metastasis of NPC cells (Cai *et al.*, 2014). Cellular miRNAs have also been shown to directly target *PTEN* including miR-21, miR-32 and miR-144, as well as miR-216a (Kato *et al.*, 2009; Meng *et al.*, 2007; Poliseno *et al.*, 2010; Wu *et al.*, 2013; Zhang *et al.*, 2013). As ovhv2-miR-73-1 shares homology with miR-216a it was hypothesised that it might also target *PTEN*.

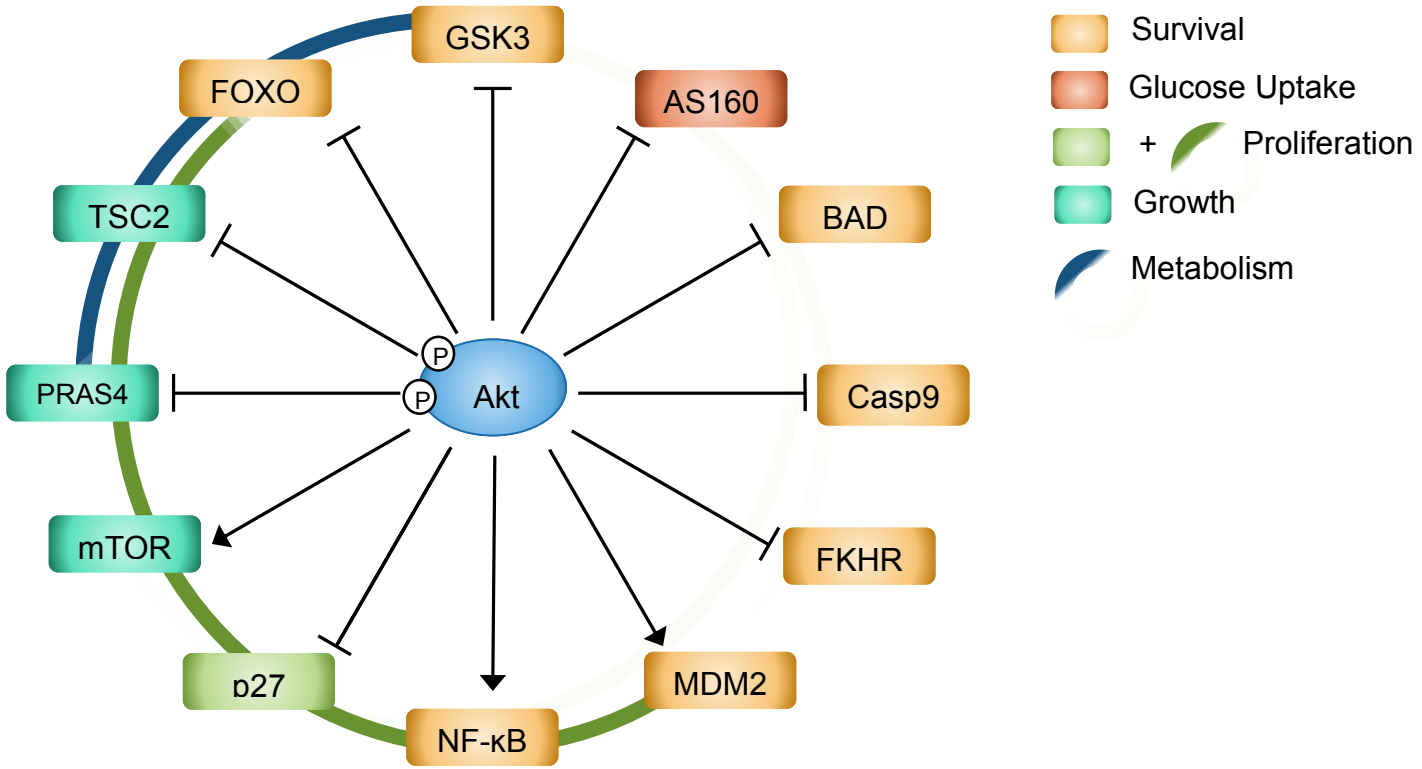


Figure 4.5 Schematic of Akt substrates and involved pathways

Phosphorylation of Akt leads to activation (arrows) or inhibition (blocking arrows) of twelve substrates shown here. The different background colours of the genes represent the different pathways they are involved in (see key for details). Connecting dark blue and dark green lines represent that the genes are involved in either metabolism or proliferation respectively.

4.4.2 YB-1 Introduction

YB-1 is a member of the cold-shock domain containing family Y-box transcription factors, first identified as a protein able to bind to the Y-box sequence present in MHC class II promoters (Mihailovich *et al.*, 2015). YB-1 can bind both DNA and RNA and is a major component of messenger ribonucleoprotein complexes (mRNPs). It plays a role in a large range of DNA- and mRNA-dependent processes including transcription, pre-mRNA splicing, translation and stabilisation of mRNA transcripts (Eliseeva *et al.*, 2015). YB-1 binds in proximity to the 5' cap structure of mRNAs and is thought to be able to compete with the translation initiation factors eIF4E and eIF4G for binding, allowing translational repression of target transcripts (Evdokimova *et al.*, 2006a). Phosphorylation of YB-1 can release transcripts returning them to the translational pool. TSC22 Domain Family, Member 1 (TSC22D1) mRNA is known to be repressed by YB-1. TSC22D1 can interact with an E-box regulator transcription factor E3 (TFE3) and in turn increase collagen type I α-2 expression (Kato *et al.*, 2010). Down-regulation of YB-1 by an OvHV-2-encoded miRNA to release *TSC22D1* transcripts could explain why sclerosis is often seen in MCF.

Research into the role YB-1 plays during virus infection has largely focused on the interaction of the human polyoma JC virus (JCV) large T antigen with YB-1 and the effect this has on the modulation of transcription of JCV-encoded genes (Safak *et al.*, 1999). There is also evidence that HIV and adenoviruses also interact with YB-1 and it has been shown that the latent membrane protein LMP2A of EBV may down-regulate YB-1 expression in gastric carcinomas (Eliseeva *et al.*, 2015; Zhang *et al.*, 2014).

Investigating the relationship YB-1 has with miRNAs reveals a 2-way interaction; cellular miRNAs can target and down-regulate *YB-1* expression, and conversely due to the RNA binding capabilities of YB-1, it has the potential to silence miRNA expression (Blenkiron *et al.*, 2013). Cellular miRNAs known to target *YB-1* include miR-216a and miR-137 (Kato *et al.*, 2010; Zhu *et al.*, 2013).

There is some crosstalk between the functions of PTEN and YB-1. YB-1 is a direct target of Akt phosphorylation which has been shown to weaken the ability of YB-1 to bind the 5' of capped mRNAs (Evdokimova *et al.*, 2006b). This phosphorylation has no effect on the RNA-binding ability of YB-1 and is thought to be a specific mechanism of Akt to increase cellular translation (Evdokimova *et al.*, 2006a).

4.4.3 RNAHybrid analysis of *PTEN* and *YB-1*

Figures 4.6 and 4.7 show the predicted binding of ovhv2-miR-73-1 and miR-216a to *PTEN* and *YB-1* in sheep (XM_004020012.2 and NM_001093786.1) and cattle (XM_613125.6 and NM_174815.2). All predicted targets are located in the 3'UTR of either *PTEN* or *YB-1*.

Analysis of the cattle *PTEN* sequence reveals one target site for both ovhv2-miR-73-1 and miR-216a; this is the site that had been previously validated for miR-216a (Kato *et al.*, 2009). The miR-216a binding site has perfect complementarity and no G:U pairing between nucleotides 2 and 8 whereas only nucleotides 2 to 7 in the ovhv2-miR-73-1 binding site share the same qualities. This difference in binding most likely explains the increase in the minimum free energy (mfe) observed for ovhv2-miR-73-1 (-13.3 kcal/mol versus -17.3 kcal/mol for miR-216a). The binding of the 3'end of ovhv2-miR-73-1 is also different to that of miR-216a, but this is expected, due to the differences in miRNA sequence between the two miRNAs. In sheep there are two binding sites for both ovhv2-miR-73-1 and miR-216a although only the site starting at position 1098 for miR-216a has been previously validated (Kato *et al.*, 2009). This site displays exactly the same binding qualities for both ovhv2-miR-73-1 and miR-216a as it does in cattle. The other site in sheep, starting at position 5815 for miR-216a, is of interest as it appears that ovhv2-miR-73-1 binds more strongly (-17.1 kcal/mol versus -14.1 kcal/mol for miR-216a).

Analysis of the cattle *YB-1* sequence showed one binding site for both ovhv2-miR-73-1 and miR-216a which is the site that had been previously validated (Kato *et al.*, 2010). Again, the miR-216a binding site has perfect complementarity and no G:U pairing between nucleotides 2 and 8 whereas for ovhv2-miR-73-1 there was only perfect complementarity and no G:U pairing between nucleotides 2 and 7. A lower mfe for ovhv2-miR-73-1 is observed (-16.3 kcal/mol versus -21.3 kcal/mol for miR-216a). The predicted targeting of ovhv2-miR-73-1 and miR-216a in sheep *YB-1* is identical to that described for cattle *YB-1*.

```
target: Cattle_PTEN
length: 3299
miRNA : ovhv2-miR-73-1
length: 23

mfe: -13.3 kcal/mol
p-value: 1.000000e+00

position  1094
target 5'   C    CA    AAUUUCAAUUU      C 3'
             UGCA   GGA           GAGAUU
             AUGU   CCU           CUCUAA
miRNA  3' UAA    UAA   CGU              U 5'


target: Cattle_PTEN
length: 3299
miRNA : miR-216a
length: 21

mfe: -17.3 kcal/mol
p-value: 1.000000e+00

position  1096
target 5' G     GAAAUU   AU        C 3'
           CACAG      UCA  UUGAGAUU
           GUGUC      GGU  GACUCUAA
miRNA  3'       AAC      C         U 5'


target: Cattle_PTEN
length: 6499
miRNA : ovhv2-miR-73-1
length: 23

mfe: -17.1 kcal/mol
p-value: 1.000000e+00

position  5814
target 5'  G     UG    UUCUCAUCU  UCUUAAAAUGGUU       U 3'
            UUACA  UGGA         GU             AGAGAUU
            AAUGU  ACCU         CG             UCUCUAA
miRNA  3' UA     UA                                 U 5'


target: Sheep_PTEN
length: 6499
miRNA : ovhv2-miR-73-1
length: 23

mfe: -13.3 kcal/mol
p-value: 1.000000e+00

position  1096
target 5'   C    CA    AAUUUCAAUUU      C 3'
             UGCA   GGA           GAGAUU
             AUGU   CCU           CUCUAA
miRNA  3' UAA    UAA   CGU              U 5'
```

```
target: Sheep_PTEN
length: 6499
miRNA : miR-216a
length: 21

mfe: -17.3 kcal/mol
p-value: 1.000000e+00

position  1098
target 5' G     GAAAUU   AU        C 3'
           CACAG      UCA  UUGAGAUU
           GUGUC      GGU  GACUCUAA
miRNA  3'       AAC      C         U 5'


target: Sheep_PTEN
length: 6499
miRNA : miR-216a
length: 21

mfe: -14.1 kcal/mol
p-value: 1.000000e+00

position  5815
target 5' U    U  GGAUUCUCAUC    CUUAAAAU    A      U 3'
           UACA GU           UGUU        GGUU GAGAUU
           GUGU CA           ACGG        UCGA CUCUAA
miRNA  3'                                        U 5'
```

Figure 4.6 RNA Hybrid analyses of Sheep and Cattle PTEN mRNA sequences for target sites of ovhv2-miR-73-1 and miR-216a.

Mfe = minimum free energy. Constraints on RNAHybrid analysis included perfect complementarity between nucleotides 2 and 8 with no G:U pairing. Positions refer to nucleotide start sites in accession numbers referenced in section 4.4.3. Targets shown are located in the 3'UTR.

```
target: Cattle_YB-1
length: 424
miRNA : ovhv2-miR-73-1
length: 23

mfe: -16.3 kcal/mol
p-value: 1.000000e+00

position  292
target 5'    C   UC   UCA   U      U 3'
              AUA  UGG   AGU GAGAUU
              UGU  ACC   UCG CUCUAA
miRNA  3' UAAA   UA         U      U 5'


target: Cattle_YB-1
length: 424
miRNA : miR-216a
length: 21

mfe: -21.3 kcal/mol
p-value: 1.000000e+00

position  291
target 5' U    UCUG   A          U 3'
           CAUA    GUC AGUUGAGAUU
           GUGU    CGG UCGACUCUAA
miRNA  3'      CAA               U 5'
```

Figure 4.7 RNA Hybrid analyses of Cattle YB-1 mRNA sequences for target sites of ovhv2-miR-73-1 and miR-216a.

Mfe = minimum free energy. Constraints on RNAHybrid analysis included perfect complementarity between nucleotides 2 and 8 with no G:U pairing. Positions refer to nucleotide start sites in accession numbers referenced in section 4.4.3. Targets shown are located in the 3'UTR.

4.4.4 Validation of *PTEN* and *YB-1* as targets of ovhv2-miR-73-1 by luciferase assay

A 100 bp sequence surrounding the target site (starting position 1096 for miR-216a in the cattle *PTEN* sequence) was cloned into psiCHECK-2 and luciferase assays were performed according to section 2.8.1 with n = 12. Figure 4.8 shows the relative luciferase expression levels for PTEN-psiCHECK-2 and YB-1-psiCHECK-2 with miR-216a and ovhv2-miR-73-1. A miR-216a mimic did not show any statistically significant difference when co-transfected with psiCHECK-2 compared to a scrambled siRNA. When a miR-216a mimic was co-transfected with a PTEN-psiCHECK-2 construct a statistically significant reduction ($p \leq 0.0001$) of 35% was observed compared to a scrambled siRNA. When an ovhv2-miR-73-1 mimic was co-transfected with the control psiCHECK-2 plasmid, a statistically significant increase in relative luciferase expression levels was observed. A statistically significant increase ($p \leq 0.01$) was also observed when an ovhv2-miR-73-1 mimic was co-transfected with a PTEN-psiCHECK-2 construct compared with a scrambled siRNA.

A 100 bp sequence surrounding the target site for ovhv2-miR-73-1 and miR-216a in *YB-1* was cloned into psiCHECK-2 and luciferase assays were performed according to section 2.8.1 with n = 12. As the 3'UTR of *YB-1* is only approximately 400 bp this was also cloned into psiCHECK-2 to determine whether the context of the target site is important in the luciferase reporter system (Appendix 2 for primer sequences). When a miR-216a mimic was co-transfected with either of the YB-1-psiCHECK-2 constructs a statistically significant reduction ($p \leq 0.0001$ for both constructs) of approximately 32-35% was observed compared to a scrambled siRNA. When an ovhv2-miR-73-1 mimic was co-transfected with the control psiCHECK-2 plasmid, a statistically significant increase ($p \leq 00001$) in relative luciferase expression levels was observed. A statistically significant increase ($p \leq 0.0001$) was also observed when an ovhv2-miR-73-1 mimic was co-transfected with a psiCHECK-2 construct containing the 3'UTR of *YB-1* compared with a scrambled siRNA. An increase in relative luciferase levels was observed when ovhv2-miR-73-1 was co-transfected with a psiCHECK-2 plasmid containing the 100 bp sequence of *YB-1* however this was not statistically significant. Due to the ovhv2-miR-73-1 mimic having a significant effect on psiCHECK-2 normalised luciferase expression it cannot be confirmed whether ovhv2-miR-73-1 targets *PTEN* and/or *YB-1* and a different validation method was required.

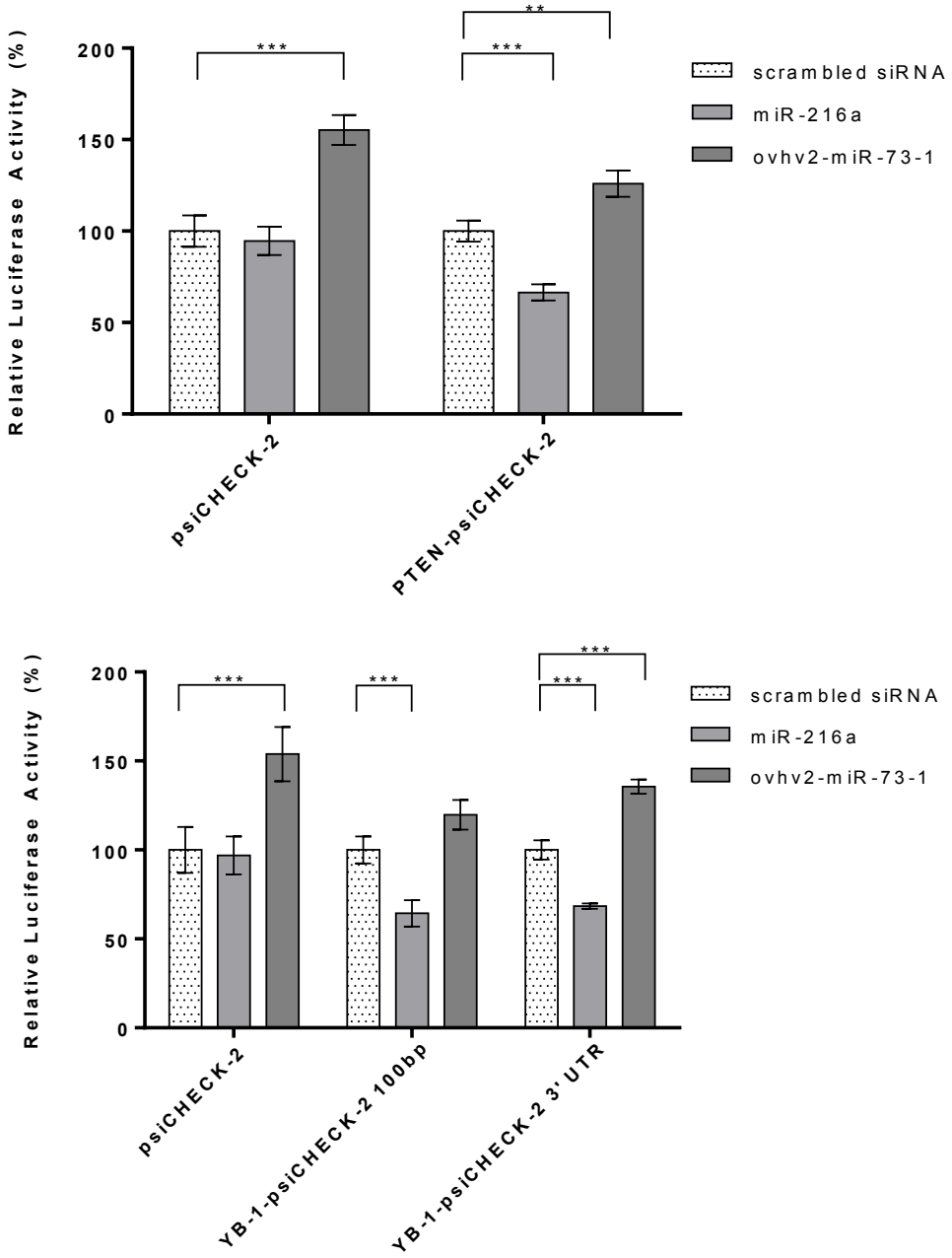


Figure 4.8 Relative luciferase expression levels of PTEN-psiCHECK-2 and YB-1-psiCHECK-2 with miR-216a and ovhv2-miR-73-1 compared to a scrambled siRNA

293T cells were co-transfected with a psiCHECK-2 construct containing a 100 bp region of PTEN (A), a 100 bp region of YB-1 or the 3' UTR of YB-1 (B), and an ovhv2-miR-73-1 mimic, a miR-216a mimic or a scrambled siRNA control. After 48 hrs, *Renilla* luciferase levels were measured, normalised to firefly luciferase levels and expression in control and test miRNAs compared. ** = $p < 0.01$, *** = $p < 0.0001$

4.4.5 Validation of *PTEN* and *YB-1* as targets of ovhv2-miR-73-1 by flow cytometry

The same 100 bp sequences of *PTEN* and *YB-1* that were used in the luciferase assays described in section 4.4.4 were also cloned into the 3'UTR of *GFP* in the GFP-expressing vector pEGFPC1. Transfections and flow cytometry were carried out as described in sections 2.1.7, 2.6.2 and 2.6.4 with n = 12 for each condition. Between replicates and conditions the percentage of GFP positive cells remained consistent (on average 31.5%, standard deviation 4.84) indicating the transfection efficiency was not altered when the EGFPC1 constructs were co-transfected with different mimics. GFP positive cells were counted and the median fluorescent intensity (MFI) for each replicate was determined and used as an indicator of GFP expression levels (Figure 4.9). When either a miR-216a mimic or an ovhv2-miR-73-1 mimic was co-transfected with EGFPC1 there was no statistically significant difference in MFI compared to a scrambled control. A statistically significant, reduction of approximately 15% for both YB-1-EGFPC1 ($p \leq 0.005$) and PTEN-EGFPC1 ($p \leq 0.001$) was observed when a miR-216a mimic was co-transfected with either plasmid compared to the scrambled siRNA control. When an ovhv2-miR-73-1 mimic was co-transfected with YB-1-EGFPC1 no statistically significant difference was observed. When an ovhv2-miR-73-1 mimic was co-transfected with PTEN-EGFPC1 a statistically significant increase ($p \leq 0.05$) in the relative MFI of EGFP expression was observed. This confirms that neither *YB-1* nor *PTEN* are targets of ovhv2-miR-73-1.

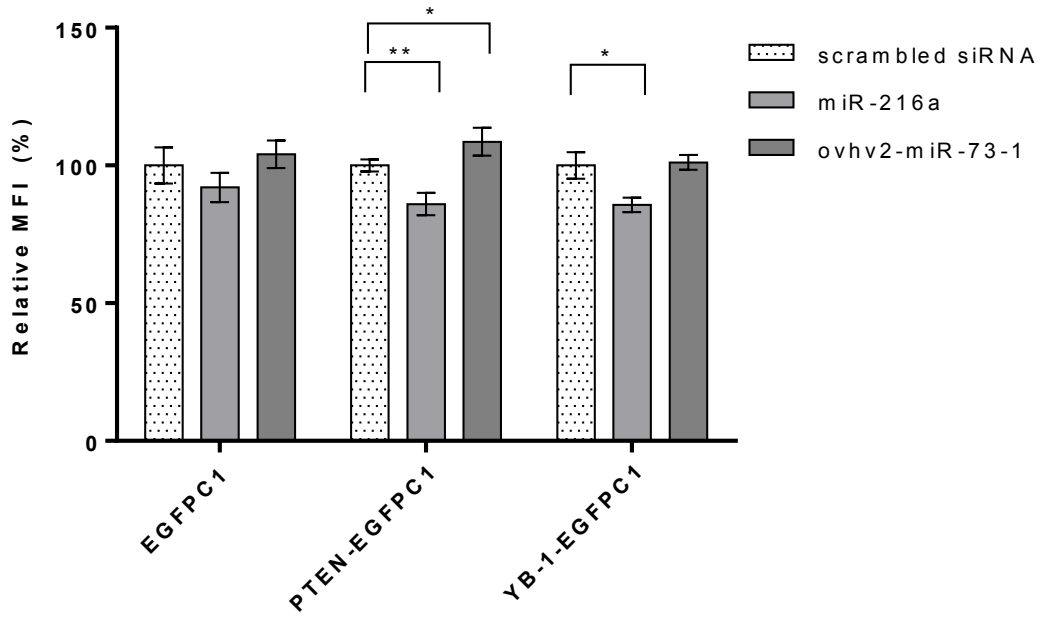


Figure 4.9 Relative median fluorescent intensity (MFI) of PTEN-EGFPC1 and YB-1-EGFPC1 with miR-216a and ovhv2-miR-73-1 compared to a scramble siRNA

293T cells were co-transfected with an EGFPC1 construct containing a 100 bp region of PTEN or a 100 bp region of YB-1 in the 3'UTR of EGFP, and an ovhv2-miR-73-1 mimic, a miR-216a mimic or a scramble siRNA control. After 48 hrs, MFI of EGFP was determined by flow cytometry and control and test miRNAs compared. * = $p < 0.05$, ** = $p < 0.001$.

4.5 Validation of MHC class II as a target of OvHV-2-encoded miRNAs

4.5.1 MHC class II

The MHC is a major component of the immune system involved in antigen presentation. MHC class II molecules are expressed on professional antigen processing cells (APCs) and exogenous antigens. Proteins captured from outside the cell from e.g. bacteria or viruses are endocytosed and processed into peptides before presentation on the surface of APCs to antigen-specific $CD4^+$ T lymphocytes (see Figure 4.10 for a simplified view of the presentation pathway). This stimulates their activation and differentiation into T helper subsets and subsequent interaction with B cells for production of antibodies against the antigen (Roche & Furuta, 2015). MHC class I molecules are expressed on all nucleated cells and present endogenous antigens on their surface. Presentation of antigens on MHC class I leads to activation of $CD8^+$ cytotoxic T lymphocytes usually associated with killing of infected cells (Hewitt, 2003).

As viruses are intracellular pathogens they are usually associated with presentation on MHC class I molecules. Herpesviruses utilise endosomal compartments for viral assembly and viral proteins are often extensively targeted to endosomes, some of these proteins will be degraded into peptides in the endosome. Therefore, herpesviruses such as EBV and KSHV that infect B cells will have viral antigens efficiently loaded and presented on MHC class II molecules (Hegde *et al.*, 2003). It has also been shown that $CD4^+$ T lymphocytes can have direct antiviral roles, usually through the production of antiviral cytokines (Hegde *et al.*, 2003; Zuo & Rowe, 2012b). It is therefore not surprising that herpesviruses modulate the MHC class II presentation pathway at a number of stages (Zuo & Rowe, 2012b) (Figure 4.10). The EBV-encoded protein BZLF1 and the KSHV-encoded protein viral interferon regulatory factor-3 (vIRF-3) both inhibit the transcription of class II MHC transactivator (*CIITA*), subsequently inhibiting the transcription of MHC class II molecules (Li *et al.*, 2009; Schmidt *et al.*, 2011). The KSHV-encoded protein ShutOff and Exonuclease protein (SOX) can degrade cellular mRNAs and as such is capable of degrading *CIITA* mRNA and MHC class II molecule mRNAs (Glaunsinger & Ganem, 2004). BZLF1 can also interfere with the normal transport and loading of peptides to MHC class II molecules through manipulation of CD74 (Zuo *et al.*, 2011). Finally, the EBV glycoprotein gp42 can abolish the interaction of the T cell receptor with MHC class II molecules thus abrogating peptide presentation to $CD4^+$ T cells (Ressing *et al.*, 2003).

BJ1035 cells are MHC class II positive (Dr Inga Dry, personal communication). Although T cells are not professional APCs and would not usually express MHC class II, a number of

immune regulators such as IFNγ can stimulate MHC class II expression. Activated human T cells have also been shown to express MHC class II molecules on their surface (Holling *et al.*, 2004). More relevantly, many sheep and cattle T cells express MHC class II on their surface (Dutia *et al.*, 1993). In line with this, there have been some reports of a reduction in the surface expression of MHC class I and class II molecules in cells supporting EBV lytic infection (Keating *et al.*, 2002). The bovine *DRB3* gene is the most widely expressed gene from the *DRB* locus, and polymorphisms associated with this gene (found in exon 2) have been associated with resistance to MCF in American bison (Das *et al.*, 2012; Traul *et al.*, 2007). As in cattle, OvHV-2-infected cells develop into LGLs that have an MHC-unrestricted phenotype, MHC class II genes are an interesting target for OvHV-2-encoded miRNAs and due to the complex nature of MHC class II, there is potential for differential regulation of MHC class II genes between sheep and cattle.

Computational analysis of a large number of immune genes looking for miRNA targets suggested that there are no human miRNAs predicted to target the 3'UTR of MHC class II genes, however miR-145 and miR-198 specifically target the 3'UTR of *CIITA* (Tomasi *et al.*, 2010). However it was also suggested by Tomasi *et al.* that *MICB* is not targeted by any human miRNAs. The CMV-encoded miRNA hcmv-miR-UL112 has been shown to target the 3'UTR of *MICB* leading to down-regulation of expression (Stern-Ginossar *et al.*, 2007). Furthermore, a KSHV-encoded miRNA, miR-K12-7, and an EBV-encoded miRNA, miR-BART2-5p have also been shown to target *MICB* (Nachmani *et al.*, 2009). This indicates that although there are no predicted human miRNAs targeting MHC class II, viruses may have the ability to target these genes, specifically for pro-viral reasons such as immune evasion.

While investigating potential targets of ovhv2-miR-73-1, BLASTN was used as a preliminary screen to investigate possible targets of this miRNA in sheep and cattle. BLASTN was used instead of RNAHybrid as a method of target identification as it is not feasible to use RNAHybrid to screen whole genomes. A large number of hits returned were in sheep MHC class II genes, with no cattle MHC class II hits (data not shown; updates to the sheep and cattle genome in NCBI now show different results than previously seen). This difference led to the hypothesis that MHC class II may be a target of OvHV-2-encoded miRNAs and that there may also be differential regulation of MHC class II genes between sheep and cattle. Down-regulation of MHC class II in sheep but not in cattle may be an important immune evasion mechanism allowing long term persistence that occurs in sheep but not in cattle.

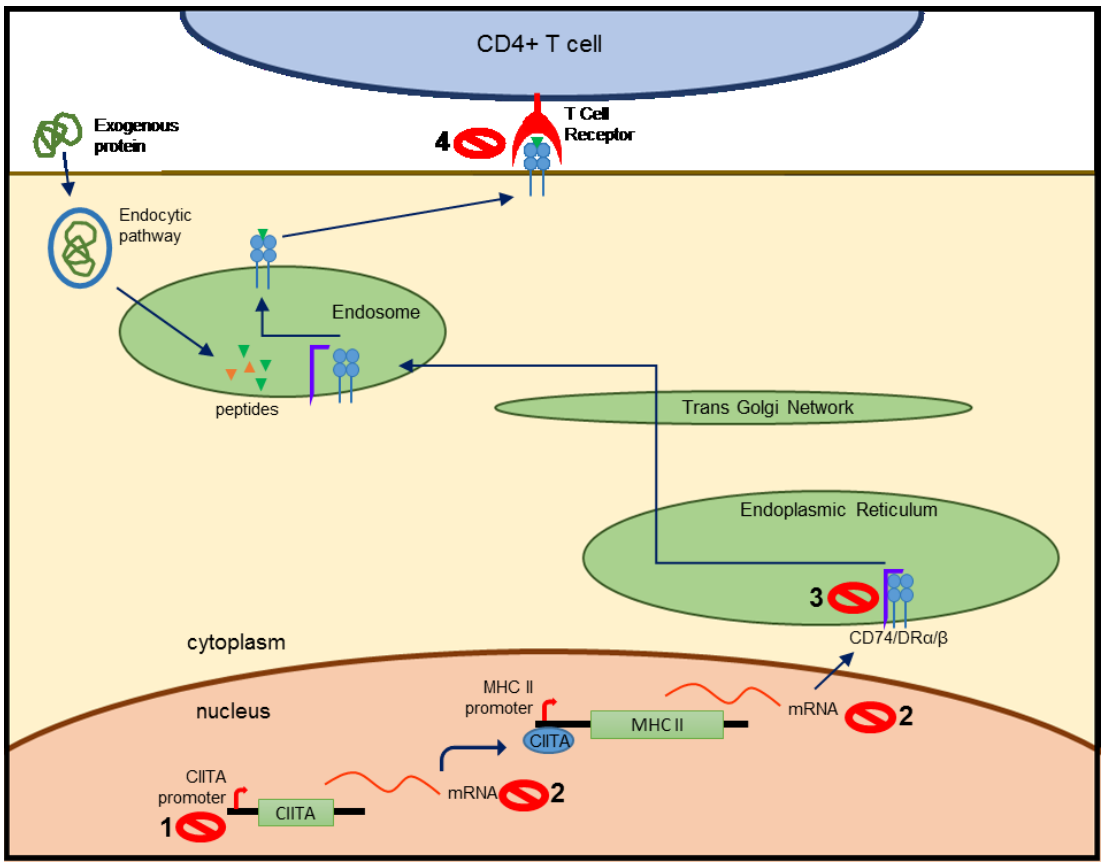


Figure 4.10 MHC class II presentation pathway and it's inhibition by EBV and KSHV

Class II MHC transactivator (CIITA) protein is made and binds to the promoter region of MHC class II genes allowing their expression. Correct folding of MHC class II proteins occurs in the endoplasmic reticulum (ER) where CD74 (also known as invariant chain) binds to prevent loading of peptides in the ER. The invariant chain facilitates transport of MHC class II proteins through the trans golgi network which fuses with late endosomes. The invariant chain is broken down by proteases and is finally removed and allows binding of peptides. Exogenous proteins can be endocytosed and degraded into peptides in endosomes and as such can be loaded onto MHC class II molecules. This pathway can be inhibited at a number of stages by EBV and/or KSHV, as indicated by red stop signs numbered 1 through 4. 1: EBV BZLF1 and KSHV vIRF3 inhibit the CIITA promoter. 2: KSHV SOX can degrade the mRNA of CIITA and MHC class II molecules. 3: EBV BZLF1 modulates CD74 interfering with transport of MHC class II molecules. 4: EBV gp42 interferes with the binding of MHC class II molecules and the T cell receptor.

4.5.2 RNA Hybrid analysis of sheep MHC class II *DQA* and *DRA* genes

Following the preliminary BLASTN screen with ovhv2-miR-73-1, RNAHybrid was used to predict target sites of ovhv2-encoded miRNAs in sheep MHC class II genes. Initially ovine *DQA* and *DRA* genes (M93430.1, M93433.1, M93431.1 and M73983.1) were investigated for potential target sites. *DQB* and *DRB* were not examined using RNAHybrid at this time. OvHV-2-encoded miRNAs were chosen for analysis if they had three or more predicted target sites in more than one gene with minimum free energies below -15 kcal/mol. Table 4.1 A lists the ovhv2-miRNAs and corresponding RNAHybrid score for each gene analysed. Full details of the RNAHybrid analysis is in Appendix 4. ovhv2-miR-17-28 has predicted binding sites in *DQA1*, *DQA2.1* and *DQA2.2*. ovhv2-miR-17-17 has two predicted binding sites in *DQA1* and one in *DRA*. ovhv2-miR-17-6 has one predicted binding site in *DQA2.2* and two in *DRA* and ovhv2-miR-17-2 has predicted binding sites in *DQA1*, *DQA2.1* and *DQA2.2*. There were no predicted target sites of ovhv2-miR-73-1 in this analysis.

Table 4.1 Summary of RNAHybrid scores for ovhv2-encoded miRNAs against sheep and cattle MHC class II genes

A: RNAHybrid scores for the four ovhv2-encoded miRNAs used in the flow cytometry assays

Gene (Ovine)	miRNA	RNAHybrid Score (kcal/mol)
DQA1	ovhv2-miR-17-28	-22.6
	ovhv2-miR-17-17	-22.7
	ovhv2-miR-17-17	-19.8
	ovhv2-miR-17-2	-24.7
DQA2.1	ovhv2-miR-17-28	-21.4
	ovhv2-miR-17-2	-28.1
DQA2.2	ovhv2-miR-17-28	-24.5
	ovhv2-miR-17-6	-23.6
	ovhv2-miR-17-2	-28.1
DRA	ovhv2-miR-17-17	-19.4
	ovhv2-miR-17-6	-23.3
	ovhv2-miR-17-6	-19.5

B: RNAHybrid scores for the ovhv2-encoded miRNAs used in the luciferase assays. See Appendix 4 for full RNAHybrid analysis.

Gene	miRNA targets	RNA Hybrid Score (kcal/mol)
Ovine *DQA*	ovhv2-miR-17-21	-24
	ovhv2-miR-17-14	-20.3
Bovine *DQA*	ovhv2-miR-17-30	-18.9
Ovine *DQB*	ovhv2-miR-17-20	-19.8
	ovhv2-miR-17-9	-25.6
Ovine *DRA*	ovhv2-miR-17-25	-19.5
	ovhv2-miR-17-17	-19.4
	ovhv2-miR-24-1	-27.6
Bovine *DRA*	ovhv2-miR-17-17	-19.4
	ovhv2-miR-24-1	-20.9
Bovine *DRB*	ovhv2-miR-17-9	-21.8

4.5.3 Flow cytometry analysis of a sheep cell line constitutively expressing MHC class II transfected with OvHV-2-encoded miRNAs

LT8.1 cells constitutively expressing ovine *DRA* and *DRB* MHC class II genes (see section 2.1.1 for details on how these cells were made) were kind gifts from Professor John Hopkins and Dr Keith Ballingall (Ballingall *et al.*, 1995). LT8.1 cells were stained with an antibody against sheep MHC class II (SW73.2), sheep IgG as an isotype control, or the secondary antibody only, as described in section 2.6.1 to check for MHC class II expression (Hopkins *et al.*, 1986). L929 cells, the cell line from which LT8.1 cells were made, was used as a negative control for all experiments described. Although the LT8.1 cells tested were positive (samples ranged from around 55% to over 90% positive depending on aliquot and passage number of cells), a fairly high percentage of positive cells was observed in cells stained with the secondary antibody alone (approximately 25%). To reduce non-specific activity of the secondary it was adsorbed against the LT8.1 cells. The secondary antibody was incubated with a 75 cm^2 flask of LT8.1 cells overnight. The next day the cells were spun at 16000 x g for 30 minutes. The supernatant containing unbound antibody was removed and stored at -80ºC. This significantly reduced the percentage of MHC class II positive cells observed in cells stained with the secondary antibody alone to levels of unstained cells. L929 cells were negative for MHC class II expression as expected.

After optimisation of the staining protocol LT8.1 and L929 cells were transfected with a range of concentrations of ovhv2-miR-73-1 or a scrambled siRNA control and monitored at 24 and 48 hrs to examine cell viability (results summarised in Table 4.2). Unexpectedly high levels of cell death were observed for both the scrambled siRNA and ovhv2-miR-73-1. At 48 hrs post transfection all of or the majority of LT8.1 cells transfected with either the scrambled siRNA or an ovhv2-miR-73-1 mimic at all of the concentrations tested were dead. At 24 hrs post transfection of LT8.1 cells the only concentration of scrambled siRNA or ovhv2-miR-73-1 that did not result in cell death was 50 nM and so these were the conditions used for flow cytometry.

LT8.1 cells were transfected in triplicate with 50 nM of ovhv2-miR--17-17, -17-6, and -73-1 or a scrambled siRNA and flow cytometry was performed 24 hrs later according to section 2.6.3 (Figure 4.11). The median fluorescent intensity (MFI) of MHC class II staining was measured and the biological replicates for each group averaged. The average MFI for each group was compared to the average MFI of the scrambled siRNA transfected cells (Figure 4.12). There was no significant difference between any of the miRNA transfected cells and scrambled siRNA transfected cells. An incubation time of 48 hrs is preferable when looking

for knockdown of protein by miRNAs and so a more suitable method of validation was required.

Table 4.2 Titration of scrambled siRNA and ovhv2-miR-73-1 in LT8.1 and L929 cells.

	24 hrs					
	untransfected	**mock**	**50 nM**	**100 nM**	**150 nM**	**200 nM**
LT8.1 scrambled	✓✓	✓✓	✓	x	x	x
LT8.1 miR-73-1	✓✓	n/a	✓	x	x	x
L929 scrambled	✓✓	✓✓	✓✓	✓	✓	x
L929 miR-73-1	✓✓	n/a	✓✓	x	x	x
	48 hrs					
	untransfected	**mock**	**50 nM**	**100 nM**	**150 nM**	**200 nM**
LT8.1 scrambled	✓✓	✓	x	x	x	x
LT8.1 miR-73-1	✓✓	n/a	x	x	x	x
L929 scrambled	✓✓	✓✓	✓✓	✓	x	x
L929 miR-73-1	✓✓	n/a	✓	x	x	x

LT8.1 or L929 cells were transfected with a range of concentrations of either a scrambled siRNA or an ovhv2-miR-73-1 mimic. At 24 hours and 48 hours cell viability was determined by visualisation of cells on a light microscope. ✓✓ = healthy cells, ✓ = cells look sick, some dead, but useable for flow cytometry and x = all or majority of cells dead and sample would not be useable for flow cytometry.

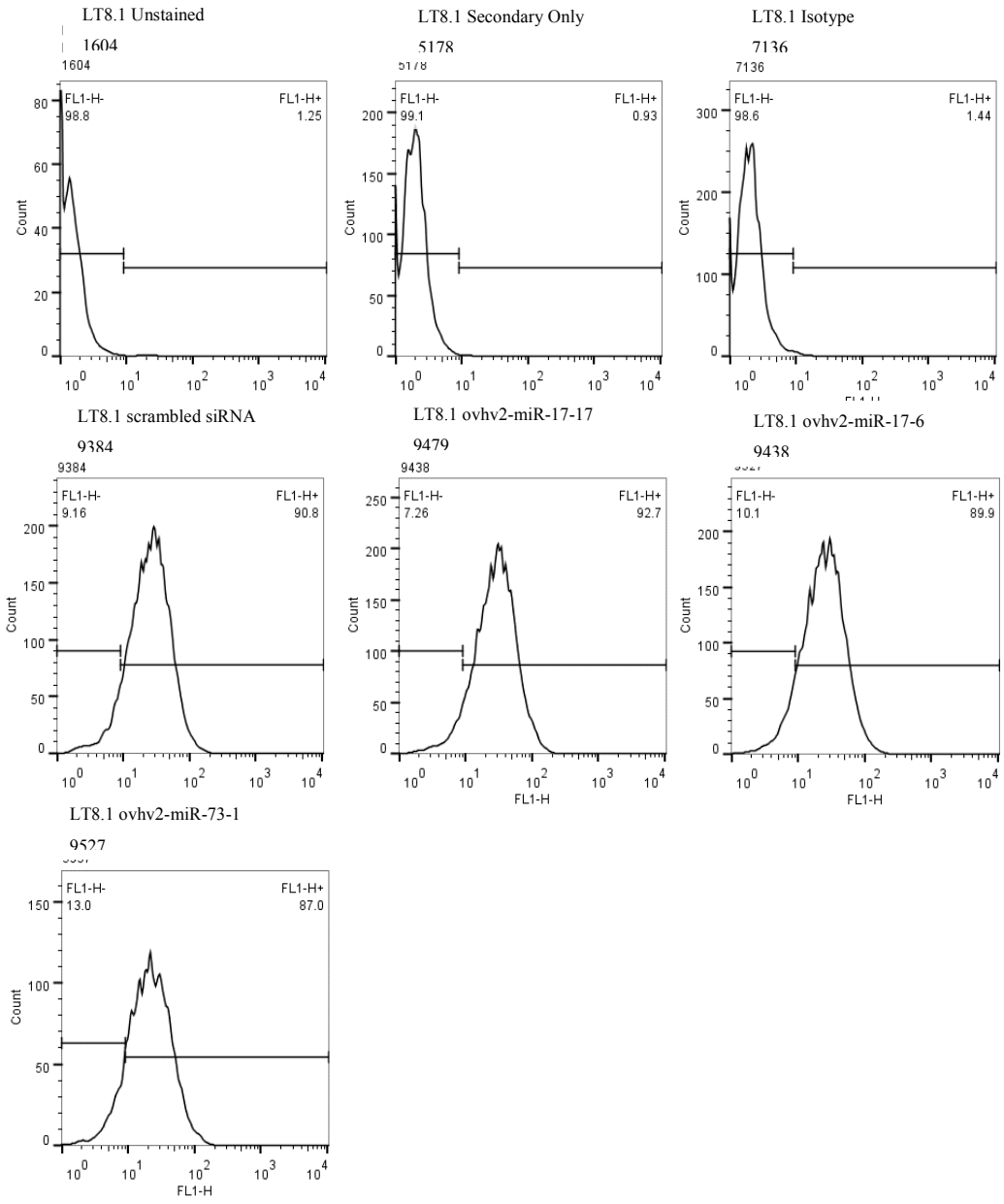


Figure 4.11 Flow cytometry analysis of LT8.1 cells transfected with 4 ovhv2-encoded miRNAs

LT8.1 cells were transfected with ovhv2-miR-17-17, -17-6, or -73-1 mimics or a scramble siRNA. Unstained cells, cells only stained with the secondary antibody and cells stained with rat anti-sheep IgG (isotype control) are shown in the top row. The number below the name of each graph represents the number of cells used in the analysis. The number in the top right of each graph shows the percentage of MHC class II positive cells. One representative data set is shown for each miRNA-transfected sample.

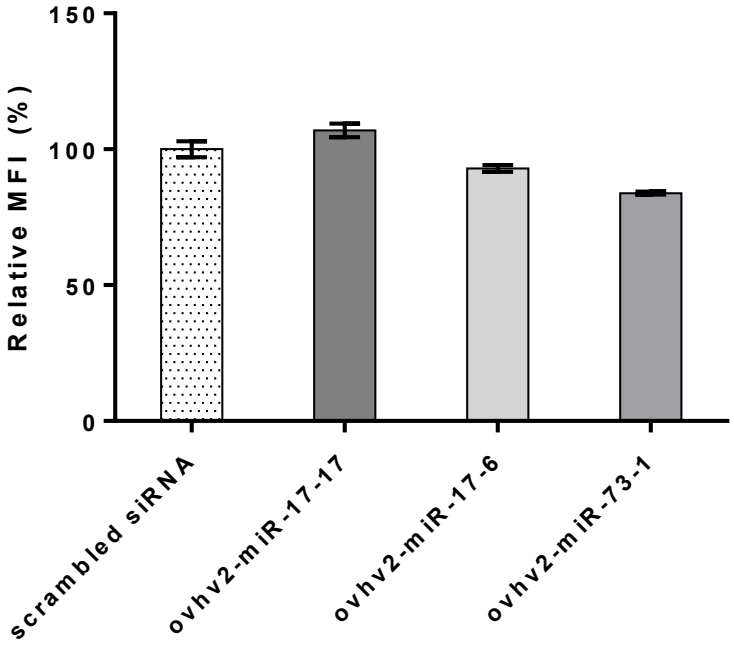


Figure 4.12 Relative MFI of MHC class II with ovhv2-encoded miRNAs or a scrambled siRNA

LT8.1 cells were transfected with ovhv2-miR-17-17, 17-6, or 73-1 mimics or a scrambled siRNA in triplicate. The MFI for each sample was determined for each sample after 24 hrs by flow cytometry and test ovhv2-miRNAs were compared to a scramble siRNA control.

4.5.4 RNA Hybrid analysis of sheep and cattle MHC class II genes

To further investigate MHC class II as a target of OvHV-2-encoded miRNAs RNAHybrid was used to analyse the 3'UTRs of both sheep and cattle MHC class II genes. Genes analysed were ovine *DQA1* (M93430.1), *DQB1* (L08792.1), *DRA* (M73983.1) and *DRB1* (M73984.1) and bovine *DQA1* (NM_001013601.3), *DQB* (NM_001034668.3), *DRA* (NM_001012677.1) and *DRB3* (NM_001012680.2). More stringent conditions were applied to this RNAHybrid analysis than in section 4.5.2, in order to decrease the number of predicted miRNA binding sites and to increase the likelihood that predicted target sites would be actual sites of OvHV-2-encoded miRNA binding. Requirements were: perfect complementarity between nucleotides 2 and 8 and no G:U pairing in this region, and an RNAHybrid score of -15 kcal/mol or lower. Table 4.1 B shows a summary of predicted OvHV-2-encoded miRNA binding sites and corresponding RNAHybrid score. Full details of the RNAHybrid analysis can be seen in Appendix 4.

There were no predicted OvHV-2-encoded binding sites for bovine *DQB* and ovine *DRB1*. Ovine *DQB* has 2 predicted binding sites, one each for ovhv2-miR-17-20 and ovhv2-miR-17-9. Bovine *DRB* has one predicted binding site for ovhv2-miR-17-9. There are two predicted binding sites (one each for ovhv2-miR-17-21 and ovhv2-miR-17-14) in ovine *DQA1* and only one predicted binding site (for ovhv2-miR-17-30) in bovine *DQA1*. The *DRA* gene is the only gene analysed in which there are binding sites for OvHV-2-encoded miRNAs in both sheep and cattle versions of the gene. ovhv2-miR-17-17 and ovhv2-miR-24-1 have one binding site each, in corresponding positions, in ovine *DRA1* and bovine *DRA1*. Ovine *DRA1* has an extra predicted binding site for ovhv2-miR-17-25. Overall these results indicate that there may be differential targeting of MHC class II genes between sheep and cattle warranting further investigation and validation.

4.5.5 Validation of sheep and cattle MHC class II as targets of OvHV-2-encoded miRNAs by luciferase assay

The 3'UTRs of ovine *DQA*, *DQB* and *DRA* and bovine *DQA* and *DRA* were cloned into psiCHECK-2 (see Appendix 2 for sequences) and luciferase assays were performed as described in section 2.8.1 with n =12 for each condition. The bovine *DRB* gene was not cloned and tested due to time constraints. For clarity, a summary table of results is shown in Table 4.3.

When an ovine DRA-psiCHECK-2 construct was co-transfected with ovhv2-miR-17-17 no statistical significance was observed compared to a scrambled siRNA control (Figure 4.13A). For ovhv2-miR-24-1 a statistically significant increase ($p \leq 0.01$) in relative luciferase

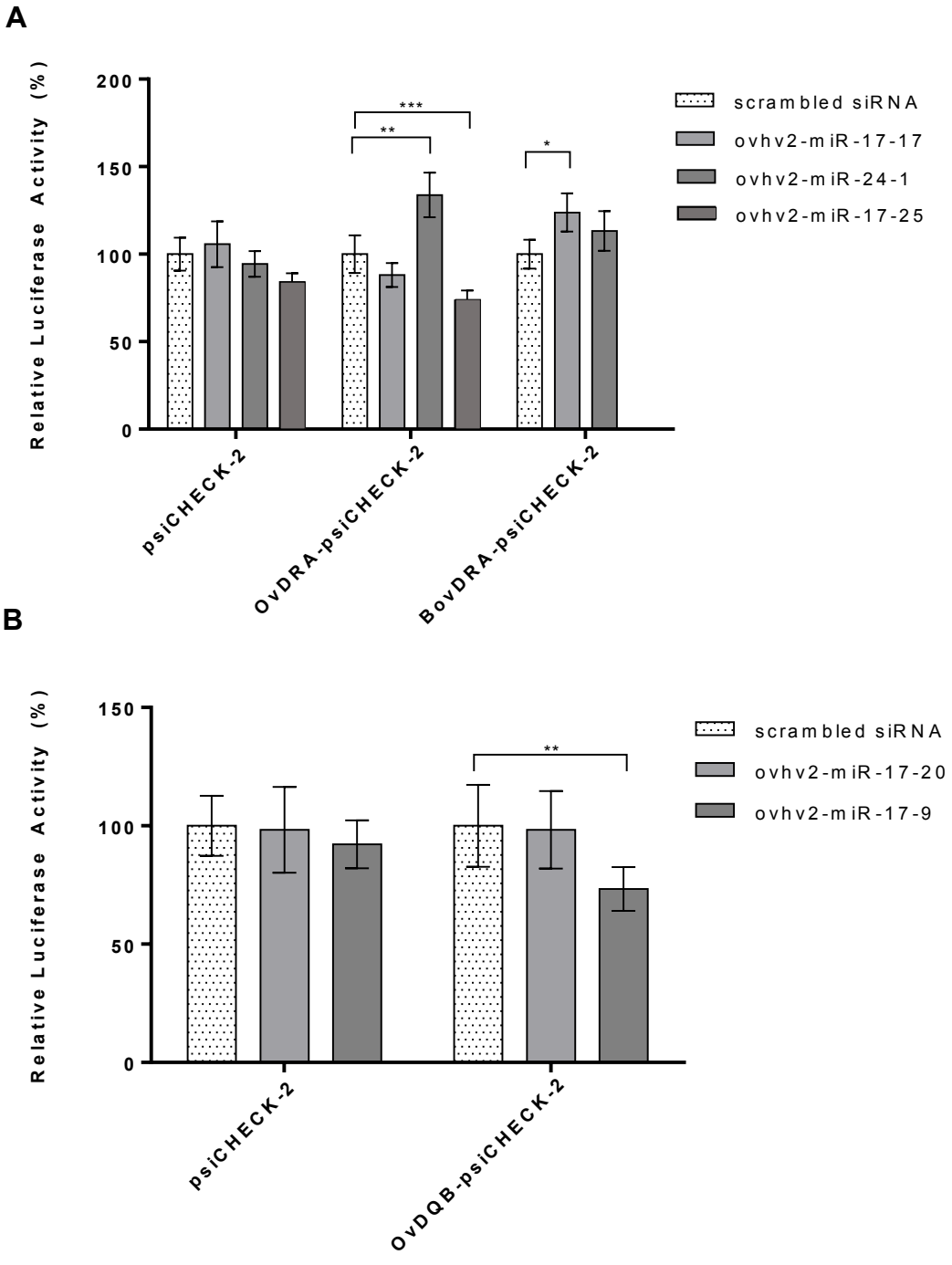
expression was observed compared to a scrambled siRNA control. A statistically significant decrease ($p \leq 0.0001$) of approximately 25% of relative luciferase expression levels was observed when ovhv2-miR-17-25 was co-transfected with the ovine DRA-psiCHECK-2 construct compared to a scrambled siRNA control. When a bovine DRA-psiCHECK-2 construct was co-transfected with either ovhv2-miR-17-17 or ovhv2-miR-24-1 an increase of approximately 25% was observed with ovhv2-miR-17-17 ($p \leq 0.05$) whereas there was no difference in relative luciferase expression levels with ovhv2-miR-24-1 compared to a scrambled siRNA control. No statistically significant changes in relative luciferase levels were observed when ovhv2-miR-17-17, miR-24-1 or miR-17-25 were co-transfected with the psiCHECK-2 construct compared to a scrambled siRNA control.

When an ovine DQB psi-CHECK-2 construct was co-transfected with either ovhv2-miR-17-20 or miR-17-9, a decrease in relative luciferase levels (approximately 25%) was only observed for ovhv2-miR17-9 ($p \leq 0.01$) compared to a scrambled siRNA (Figure 4.13 B). No significant difference was observed for ovhv2-miR-17-20 compared to a scrambled siRNA control. No significant difference was observed when either mimic was co-transfected with psiCHECK-2 compared to a scrambled siRNA.

When ovhv2-miR-17-30, 17-21 and 17-14 were co-transfected with psiCHECK-2, significant decreases of 68% ($p \leq 0.0001$), 55% ($p \leq 0.0001$) and 37% ($p \leq 0.01$) in relative luciferase levels were observed respectively compared to a scrambled siRNA (Figure4.13 C and D). Although a significant decrease ($p \leq 0.0001$) in relative luciferase expression levels was observed when ovhv2-miR-17-30 was co-transfected with a bovine DQA-psiCHECK-2 construct it cannot be confirmed whether this decrease is due to direct targeting of bovine *DQA* and further investigation would be needed. No significant difference was observed when ovhv2-miR-17-21 was co-transfected with an ovine DQA-psiCHECK-2 construct compared to a scrambled siRNA. A statistically significant decrease ($p \leq 0.05$) in relative luciferase expression levels when ovhv2-miR-17-14 was co-transfected with and ovine DQA-psiCHECK-2 construct was observed compared to a scrambled siRNA, however this decrease was smaller than that observed for psiCHECK-2 alone thus making it unlikely that ovhv2-miR-17-14 targets ovine *DQA*.

In summary, transfection with 2 OvHV-2-encoded miRNAs cause statistically significant decreases of approximately 25% in relative luciferase expression compared to a scrambled siRNA; ovhv2-miR-17-25 with ovine DRA-psiCHECK-2 and ovhv2-miR-17-9 with ovine DQB-psiCHECK-2. ovhv2-miR-17-30 may target bovine *DQA* however this could not be confirmed due to an observed decrease with this miRNA in the control plasmid. To confirm

these results a second method of validation would be required and due to time constraints this work was not continued.



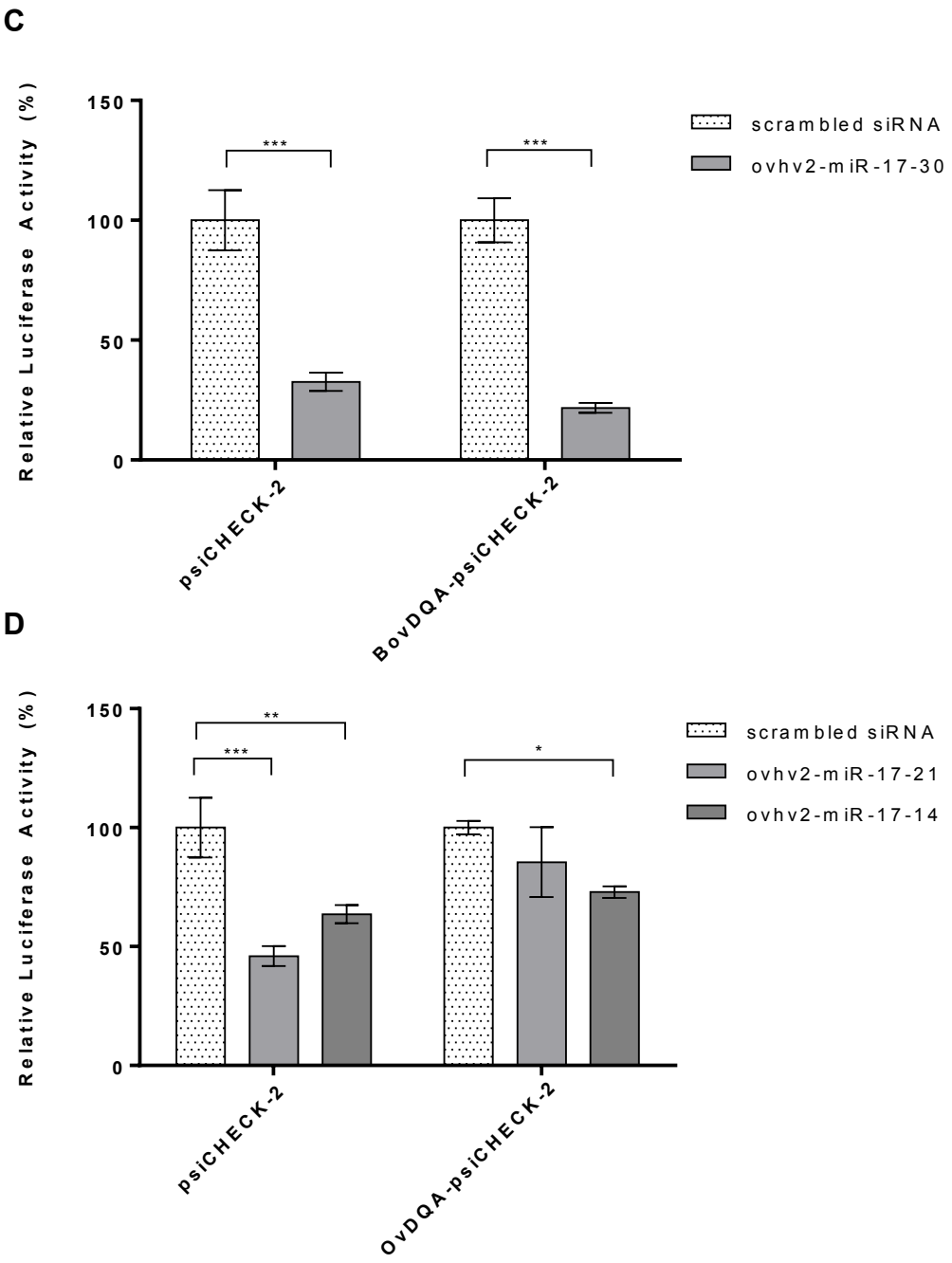


Figure 4.13 Relative luciferase expression levels of MHC class II psiCHECK-2 constructs with ovhv2-encoded miRNAs compared to a scrambled siRNA

293T cells were co-transfected with psiCHECK-2 constructs containing the 3'UTRs of ovine DRA (A), DQB (B) and DQA (D) and bovine DRA (A) and DQA (C) and ovhv2-encoded miRNAs with predicted targets in the region (Table 4.1). After 48 hrs, *Renilla* luciferase levels were measured, normalised to firefly luciferase levels and expression in control and test miRNAs compared. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$

Table 4.3 Summary of MHC class II luciferase assay results

Plasmid	Ovhv2-miRNA	Effect	Statistically significant?
psiCHECK-2	ovhv2-miR-17-17	None	No
	ovhv2-miR-24-1	None	No
	ovhv2-miR-17-25	None	No
	ovhv2-miR-17-20	None	No
	ovhv2-miR-17-9	None	No
	ovhv2-miR-17-30	Down	Yes
	ovhv2-miR-17-21	Down	Yes
	ovhv2-miR-17-14	Down	Yes
Ovine *DRA*	ovhv2-miR-17-17	Down	No
	ovhv2-miR-24-1	Up	Yes
	ovhv2-miR-17-25	Down	Yes
Bovine *DRA*	ovhv2-miR-17-17	Up	Yes
	ovhv2-miR-24-1	Up	No
Ovine *DQB*	ovhv2-miR-17-20	None	No
	ovhv2-miR-17-9	Down	Yes
Bovine *DQA*	ovhv2-miR-17-30	Down	Yes
Ovine *DQA*	ovhv2-miR-17-21	Down	No
	ovhv2-miR-17-14	Down	Yes

4.6 Discussion

4.6.1 DLL1

Validation of *DLL1* as a target of ovhv2-miR-17-2 has been complicated by the incorrect annotation of the sheep genome assembly OARv3.1. Initial luciferase assays showed that ovhv2-miR-17-2 does target *DLL1*, with a reduction in relative luciferase expression of approximately 35%. Mutation of either site 1 or site 3 did not restore relative luciferase expression levels comparative to the scrambled siRNA however when both sites were mutated relative luciferase expression was restored. This implies that, in the luciferase reporter system, there is some redundancy of target sites and just one site is sufficient for reduction in relative luciferase expression.

The major problem encountered in the validation of *DLL1* was that the published sequence of sheep *DLL1* in both NCBI and Ensembl was incorrect. PCR and sequencing of the region predicted to contain the three target sites revealed that sites 1 and 2 are not present in this region. The published sheep *DLL1* mRNA sequence does not align to the cattle *DLL1* mRNA sequence in this region however the sequence obtained by PCR performed in section 4.3.5 aligned well to the cattle *DLL1* mRNA sequence. Conservation of the sequence of *DLL1* between species is high (Appendix 3 for alignments) and so it was surprising that in sheep there was a high level of variation in the published sheep *DLL1* sequence compared to the cattle sequence. Further PCRs including 5' and 3' RACE were performed in order to try and identify where in the sheep genome target sites 1 and 2 mapped; whether it was a different region of *DLL1* or an entirely different gene not annotated in the sheep genome. Unfortunately 5' RACE did not yield any interesting results; none of the clones sequenced contained the primer sequence and were therefore excluded from further analysis. 3' RACE was successful in that clones sequenced contained the primer sequence; the sequences obtained mapped to *DLL1* sequences in closely related species such as goat and cattle; and the sequence obtained contained the complete 3'UTR. Frustratingly, target sites 1 and 2, which should have been adjacent to the primer sequence, were absent. As 3' RACE amplifies from cDNA, and the 3 target sites are predicted to be present in two consecutive exons, genomic DNA was sequenced to include the intron between the two exons. It could have been possible that the sequence identified in CLASH was an unusual transcript produced by alternative splicing, especially as the sequence of the intron was not defined. The results of this sequencing showed that this intron did not exist which, is again in line with the cattle *DLL1* sequence. So although it was confirmed that ovhv2-miR-17-2 does target site 1, the identity of the gene remains unknown. Steps described above were taken to try and elucidate the identity of the target gene but this

was not successful. A BLASTN analysis of the hybrid sequence obtained from the CLASH study was performed against the sheep genome, the nucleotide collection database, the reference RNA database, the NCBI genomes databse and the expressed sequence tags database. The only match from these analyses was the sheep *DLL1* sequence, which is clearly incorrect. The sequence did not correspond to any other annotated sheep gene. Further investigation into the location of site 1 was deemed to be too time consuming and more rewarding aspects of OvHV-2-encoded miRNA target identification were pursued.

As target site 3 had definitely been confirmed to be present in the *DLL1* sequence further luciferase assays carried out containing this sequence demonstrated a smaller, but still significant knockdown of approximately 25%. Mutagenesis of this site to confirm that site 3 is the only target of ovhv2-miR-17-2 binding was not carried out due to time constraints. Analysis of differential targeting between sheep and cattle at this site was also not carried out due to time constraints, Comparison of the RNAHybrid scores for this site between the sheep and cattle *DLL1* sequence indicate that targeting would be very similar. It is therefore unlikely that targeting of *DLL1* plays a role in the different outcomes of virus infection between sheep and cattle.

Further validation of *DLL1* as a target of ovhv2-miR-17-2 by a different method was hindered by the lack of sheep and cattle cell lines that expressed *DLL1*. Although BJ1035 cells did express *DLL1*, the expression was below the level of accurate quantification, and as such was not an appropriate system to study *DLL1* knockdown (data not shown); RT-qPCRs to examine knockdown of natively expressed *DLL1* by ovhv2-miR-17-2 could therefore not be carried out.

As DLL1 is a ligand and Notch signalling is cell-cell mediated it would be difficult to characterise the phenotypic changes seen in Notch signalling due to a down-regulation of *DLL1*. Additionally, there is no guarantee that an infected cell will come into contact with another infected cell thus altering the phenotype of infected cells. However, it is possible that the environment infected cells are in could alter the infected cell phenotype, for example through cytokine signalling. As mentioned in section 4.3.1, *GATA3* expression can be induced by Notch, which can then induce the expression of *IL-4* (Fang et al., 2007). IL-4 is a pleiotropic cytokine known to play a role in the promotion of the Th2 cell lineage (Seder *et al.*, 1992). IL-4 signals through signal transducer and activation of transcription 6 (STAT6). It has been shown that in B cells, STAT6 is capable of up-regulating MHC class II expression (Takeda *et al.*, 1996). Therefore, it is possible that a reduction in activation of Notch signalling through reduced expression of DLL1 could result in lower IL-4 signalling and a reduction in expression

of MHC class II. This may contribute to the immune evasion which is likely required for successful latent infection of OvHV-2. Alternatively, in the context of MCF, a reduction in IL-4 signalling could have the effect of altering the balance of Th1/Th2 cells in the infected cell environment, with a potential polarisation towards Th1 cells. Polarisation towards Th1 cells has the potential to result in inflammatory or autoimmune disease (Wurster *et al.*, 2000). As MCF is a lymphoproliferative disease characterised by MHC-unrestricted cytotoxicity it could be somewhat described as an autoimmune disease and thus altered Notch signalling could be a contributing factor to the pathogenesis observed in affected animals. However, it is unlikely that this would be on the only factor, and a number of other modulations of the environment and the infected cell would be required to result in disease.

Other OvHV-2-encoded miRNAs were predicted to target sheep and cattle *DLL1* and as such differential targeting could still exist. However the sheep sequence of *DLL1* is obviously incorrectly annotated and would most likely require further sequencing before confidence in RNAHybrid predictions could be gained. Although an interesting target with high relevance for OvHV-2 infection and MCF pathogenesis it was decided to not continue the validation of *DLL1* as a target of ovhv2-miR-17-2.

4.6.2 PTEN and YB-1

It was demonstrated that, despite having homology with mir-216a, ovhv2-miR-73-1 does not target *PTEN* and *YB-1*. As seen in Figure 3.1 the first 7 nucleotides of ovhv2-miR-73-1 are identical to miR-216a. The seed sequence of the miRNA has been thought to be most important in binding mRNA. Recent work has identified that perfect complementarity between the seed sequence of miRNA and target mRNA is not necessary for down-regulation of target mRNA (Helwak *et al.*, 2013). Moreover, non-canonical binding may be much more widespread than first thought and this will be under represented by miRNA target prediction programmes that focus on perfect base pairing in the seed sequence (Helwak *et al.*, 2013). In this case however it appears that canonical miRNA binding is the main determinant of *PTEN* and *YB-1* knockdown. The RNAHybrid predictions of ovhv2-miR-73-1 binding to *PTEN* and *YB-1* mRNA are of a lower mfe than miR-216a and do not have perfect complementarity between nucleotides 2 and 8. It is therefore likely that RNAHybrid is a good predictor of canonical miRNA binding. The other ovhv2-miR-73-1 and miR-216a binding site for *PTEN* in sheep discovered by RNAHybrid was not analysed due to time constraints. Limitations of RNAHybrid will be discussed in chapter 6.

The major problem encountered in this section concerned the effect of ovhv2-miR-73-1 on the psiCHECK-2 vector. A significant increase in relative luciferase levels was consistently and

reproducibly observed when ovhv2-miR-73-1 was co-transfected with the empty psiCHECK-2 vector compared to a scrambled siRNA control. RNAHybrid was performed on the psiCHECK-2 plasmid and no ovhv2-miR-73-1 binding sites were present in the coding region of either firefly luciferase or *Renilla* luciferase. This indicated that there was no off-target effects of ovhv2-miR-73-1 on the psiCHECK-2 plasmid. Attempts to eliminate this effect by changing parameters such as concentration of vector and mimic, incubation time, brands of mimics and cell type were unsuccessful. It was concluded that for this mimic luciferase assay was not an appropriate system for target validation and another system was required for validation. The kinds of off-target effects observed here have not been described in the literature. Furthermore, the effect of ovhv2-miR-73-1 in this assay may not be directly linked to a downregulation of luciferase and could be an un-identifiable artefact of the assay. More generally, few off-target effects have been described and are usually limited to the attempt to reduce off-target effects in the design of small interfering RNAs or short hairpin RNAs or in the use of miRNAs as therapeutics (Aleman *et al.*, 2007; Jackson *et al.*, 2006; Ji Diana Lee *et al.*, 2015; Singh *et al.*, 2011)

A GFP reporter system was used as an alternative method for validation. When miR-216a was co-transfected with a GFP plasmid containing *PTEN* or *YB-1* sequences the level of knockdown observed was smaller than in the luciferase reporter system but still statistically significant. The difference in knockdown observed is most likely due to variation of the 2 different systems used. The problems observed with ovhv2-miR-73-1 in the luciferase reporter system were not seen in the GFP reporter system. It can therefore be concluded that, at least using a GFP reporter system, ovhv2-miR-73-1 does not target *PTEN* or *YB-1*.

4.6.3 MHC class II

Investigation into whether MHC class II is targeted by OvHV-2-encoded miRNAs is complicated by the fact that in sheep and cattle, MHC class II is a complex of four genes, *DQA/B* and *DRA/B*. Sheep *DQA* and *DRA* genes were analysed first as the LT8.1 cell line that constitutively expresses sheep MHC class II genes was readily available. No restrictions on binding dynamics were imposed in this initial RNAHybrid analysis, however to be taken forward for further analysis miRNAs had to target multiple genes. It should be noted that the initial interest in MHC class II arose from a BLASTN analysis of the seed sequence of ovhv2-miR-73-1 against the sheep and cattle genome but that RNAHybrid did not predict any ovhv2-miR-73-1 target sites in either sheep or cattle MHC class II genes.

Transfection of LT8.1 and L929 cells was complicated by severe mimic toxicity, limiting both concentration of mimic and incubation period used when using flow cytometry as a method of

assessing knockdown of MHC class II by OvHV-2-encoded miRNAs. As this toxicity was observed with the scrambled siRNA as well as ovhv2-miR-73-1 it is likely that this is a cellular response to dsRNA transfection. L929 cells have been shown to be sensitive to TNFα (Humphreys & Wilson, 1999). TNFα can be induced by the dsRNA recognition molecule PKR (Takada *et al.*, 2006; Yeung *et al.*, 1996). It is possible that PKR can recognise the transfected mimics and induce a TNFα response leading to apoptosis. This mimic toxicity meant that a lower mimic concentration had to be used with a shorter incubation period. No difference in the MFI of MHC class II was observed in any of the OvHV-2-encoded miRNA mimic transfected cells compared to those transfected with a scrambled siRNA. As the transfection protocol had to be compromised to alleviate toxicity it could not be confirmed whether OvHV-2-encoded miRNAs target MHC class II and another approach was required.

The luciferase reporter system was implemented as a method of screening MHC class II genes for targets. As the luciferase reporter system included more MHC class II genes more stringent parameters were applied to this second RNAHybrid analysis than the first RNAHybrid analysis used for the flow cytometry analysis. This resulted in different OvHV-2-encoded miRNAs identified with potential binding sites in the MHC Class II genes analysed. Of the eight OvHV-2-encoded miRNAs chosen for analysis, three (ovhv2-miR-17-30, ovhv2-miR-17-21 and ovhv2-miR-17-14) displayed a statistically significant down-regulation of relative luciferase expression levels in the psiCHECK-2 plasmid alone compared to the scrambled siRNA. When RNAHybrid was performed on ovhv2-miR-17-21 against the psiCHECK-2 sequence no binding sites were predicted, even allowing for G:U pairing in the seed region. When RNAHybrid was performed on ovhv2-miR-17-30 and ovhv2-miR-17-14 a number of binding sites in the firefly luciferase and *Renilla* luciferase sequences were predicted when G:U pairing in the seed region was allowed (Appendix 5). ovh2-miR-17-30 has six predicted sites in the firefly luciferase sequence and five in the *Renilla* luciferase sequence. ovhv2-miR-17-14 has two predicted targets sites in both the firefly luciferase and *Renilla* luciferase sequences. Predicted sites may not be functional target sites and so it could be possible that the predicted sites in *Renilla* luciferase are functional whereas those in firefly luciferase are not functional. If this is true, then down-regulation of relative luciferase levels observed when ovhv2-miR-17-30 and ovhv2-miR-17-14 are co-transfected with psiCHECK-2, is due to off target effects of these mimics.

The other five OvHV-2-encoded miRNA mimics showed no significant difference in relative luciferase expression levels of the psiCHECK-2 plasmid alone compared to the scrambled siRNA. This allowed for the identification of two OvHV-2-encoded miRNAs, ovhv2-miR-17-

25 and ovhv2-miR-17-9 that target sheep *DRA* and sheep *DQB* respectively. A third miRNA, ovhv2-miR-17-30 may target cattle *DQA* although, due to problems with the luciferase assay this could not be confirmed.

The results presented here support the hypothesis that MHC class II may be down regulated in sheep as an immune evasion strategy and promoting and allowing the maintenance of latency. As outlined in section 4.5.1 many herpesviruses modulate the MHC class II signalling pathway as a mechanism of immune evasion, however these results are the first to demonstrate the potential for miRNA-mediated regulation of MHC class II. From these results, it appears that there may be differential targeting of MHC class II between sheep and cattle. It is therefore possible that MHC class II signalling is different between infected sheep and cattle leading to altered outcomes of latency establishment. However, it is unlikely to be just a single factor that results in the development of disease in one animal and not in another and more likely multiple factors acting on multiple different pathways. It is therefore unclear how targeting of MHC class II in cattle contributes to MCF pathogenesis, and any possible mechanism would be speculation, given the preliminary nature of this data. Further work, such as mutagenesis of the predicted target sites would be required to confirm the targeting, and use of another method of validation such as the GFP reporter system described for *PTEN* and *YB-1* would be ideal.

Other methods of validation and characterisation of these interactions could include development of a RT-qPCR and use of sheep and cattle lymphocytes to look at miRNA knockdown in a biologically relevant cell line. It would be important to test how knockdown of MHC class II genes at an RNA or protein level would affect surface expression levels of MHC class II over time. Time constraints on the project did not allow for full characterisation of miRNA knockdown and so any downstream experiments could not be followed up. Other OvHV-2-encoded miRNA targets were giving more promising results and so this work was not continued.

Chapter 5: Identification of the virus protein Ov2 as a target of OvHV-2-encoded miRNAs

5.1 Introduction

There are many documented examples of regulation of herpesvirus gene expression through expression of their own miRNAs (see section 1.7.2 for examples). These virus-encoded miRNAs often play roles in the maintenance of latency and pathogenesis of the virus. It has previously been shown by the Dalziel group that OvHV-2 encoded ORF50 and ORF73 are targets of ovhv2-miR-17-1 and 17-10 respectively (Riaz *et al.*, 2014). ORF50 and ORF73 are two of the most well characterised genes in the gammaherpesvirus subfamily and the roles they play in virus biology are well defined. However, there are a number of genes unique to OvHV-2 and AlHV-1 (see section 1.5.1), the functions of which are less well characterised and it is therefore unknown what roles they play, if any, in pathogenesis. One of these proteins, Ov2, is predicted to contain a basic leucine zipper domain (bZIP domain). bZIP domains are bipartite; they have a basic domain that is capable of interacting with the DNA backbone and a leucine zipper motif (Vogt, 2001). The leucine zipper motif is characterised by heptad repeats of leucine residues that form an amphipathic helix (Landschulz *et al.*, 1988). Upon dimerization with another protein containing a bZIP domain, a coiled coil conformation occurs with the leucine residues aligned along the contact surface (Rasmussen *et al.*, 1991).

Proteins with bZIP domains are often transcription factors. Activator protein 1 (AP-1) is a dimeric complex that contains members of the JUN, FOS, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma (MAF) protein families (Eferl & Wagner, 2003). Dimerization of bZIP proteins is required for functional activity; many of these proteins can form homodimers and/or heterodimers however some, like the FOS family, cannot form homodimers. The specificity of dimerization depends on the non-leucine residues of the zipper domain (Alber, 1992). Different combinations of homodimers and heterodimers can form AP-1 and the combination of proteins in the dimer determines AP-1 function and which genes are regulated. The JUN family consists of c-JUN (originally named after the viral counterpart v-Jun in avian sarcoma virus 17), JUNB and JUND (Lopez-Bergami *et al.*, 2010; Maki *et al.*, 1987). The FOS family consists of c-FOS (originally named after the viral counterpart v-Fos in Finkel-Biskis-Jinkins osteosarcoma virus), FOSB, FOS-related antigen 1 (FRA1) and FRA2 (Angel & Karin, 1991; Curran *et al.*, 1982). c-FOS, FOSB and c-JUN all have potent transactivator domains; overexpression of these proteins leads to efficient transformation of cells in culture. Thus, they are defined as oncoproteins and therefore AP-1 can play an important role in tumorigenesis (Jochum *et al.*, 2001). AP-1 is thought to regulate the expression of genes involved in all of the six "hallmarks of cancer" (sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death) (Eferl & Wagner,

2003; Hanahan & Weinberg, 2000; Lopez-Bergami *et al.*, 2010; Shaulian & Karin, 2002). A c-JUN-FRA1 heterodimer leads to up-regulation of miR-21 expression, a key oncogene highly expressed in many cancers that is known to down-regulate tumour suppressors such as PTEN (Melnik, 2015). Conversely, AP-1 proteins that lack a potent transactivator domain can either have weak transforming potential (FRA1 and FRA2) or can have tumour suppressor effects (JUNB and JUND) (Bergers *et al.*, 1995; Deng & Karin, 1993; Foletta *et al.*, 1994; Pfarr *et al.*, 1994). AP-1 can therefore have tumour promoting or tumour suppressing activity depending on the different combinations of proteins, however AP-1 activity is probably also influenced by type, stage and genetic background of the tumour (Eferl & Wagner, 2003).

The potential for bZIP proteins to interact with a wide number of proteins that can influence gene expression on a large scale makes them an attractive choice for a herpesvirus that needs to manipulate the cellular environment with minimal coding capacity. Ov2 is therefore an interesting protein to study as it may have significant effects on the pathogenesis of OvHV-2 and could also potentially interact with different cellular bZIP proteins between sheep and cattle. There is no published data available about the function of Ov2. One study on A2, the positional homologue of Ov2 in AlHV-1, has been published which shows A2 has an effect on the cellular transcriptome and cytotoxicity of LGLs in infected rabbits (Parameswaran *et al.*, 2014). In this study an A2 gene knockout AlHV-1 was produced and rabbits were infected with this virus, the wild-type, or a revertant A2 virus. All the infected rabbits succumbed to MCF, however the onset of MCF in rabbits infected with the A2 knockout virus was delayed compared to the wild-type or revertant viruses. This indicated that A2 is not a critical virulence factor for AlHV-1. Infected LGLs were obtained from infected rabbits and RNA-Seq was performed to identify differentially expressed genes. A2 was found to be involved in the transcriptional regulation of genes involved in immunological, cell cycle and apoptosis pathways. Partial inhibition of the cytotoxicity-associated pathways involving perforin and granzymes A and B (proteins found in cytotoxic granules) was observed in the A2 knockout virus compared to the controls indicating that A2 plays a role in enhancing the cytotoxicity in LGLs (Parameswaran *et al.*, 2014).

Positional homologues of herpesvirus-encoded genes can sometimes have shared or similar functions, especially in more conserved ORFs such as ORF73 in KSHV and MHV-68 and EBNA1 in EBV, which share functions. Positional homologues of Ov2 in other gammaherpesviruses include LMP-1 in EBV, K1 in KSHV, STP/Tip in HVS and M1 in MHV-68 (Krug *et al.*, 2013; Raab-Traub, 2012; Tsygankov, 2005; Wang *et al.*, 2004a). LMP-1, K1 and STP/Tip are all membrane proteins that play a role in virus-induced transformation of

cells. M1 is a secreted protein with superantigen-like activity that can lead to an increased establishment of latency in a particular subset of B cells (Krug *et al.*, 2013). As none of these proteins contain a bZIP domain it is unlikely that there will be any shared functions between Ov2 and these other positional homologues.

Other gammaherpesviruses encode bZIP proteins including EBV and KSHV, which encode BZLF1 and K8 respectively (described in more detail in section 1.3.2). MDV encodes the bZIP protein Meq, which is highly expressed in virus-induced lymphoblastoid tumours (Jones *et al.*, 1992). Meq resembles the FOS/JUN family of bZIP proteins and is capable of transforming chicken cells via the v-Jun transcriptional cascade (Levy *et al.*, 2005). Meq is a target of the MDV-encoded miRNA miR-M4 and it has been shown that this miRNA is critical for the induction of lymphomas since either deletion of the miRNA or mutation of the seed region abolished the ability of MDV to induce lymphomas *in vivo* (Burnside *et al.*, 2006; Zhao *et al.*, 2011).

5.2 Aims

The aims of this part of the project were to use bioinformatic analysis to investigate whether any OvHV-2-encoded miRNAs had potential binding sites in Ov2, and then to validate the predicted target sites using biochemical methods. Finally, as the function of Ov2 is currently unknown, perform functional analysis of Ov2 to determine subcellular localisation, identify genes that are potentially regulated by Ov2 and elucidate potential interacting partners.

5.3 Validation of Ov2 as a target of OvHV-2-encoded miRNAs

5.3.1 RNAHybrid analysis of Ov2 mRNA sequence

RNAHybrid was used to investigate the Ov2 mRNA sequence (AY839756.1, reverse complement of nucleotides 1321 – 2480) for targets of OvHV-2-encoded miRNAs. Analyses of coding region targets can be seen in Fig. 5.1. A further four targets were identified in the 3'UTR of Ov2 but were not taken forward for further validation (see Appendix 9). Targets identified by RNAHybrid had perfect complementarity between nucleotides 2 and 8 of the miRNA and no G:U base pairing in that region. Four OvHV-2-encoded miRNAs were shown to have targets in the coding region: ovhv2-miR-17-29, ovhv2-miR-17-10, ovhv2-miR-61-1 and ovhv2-miR-73-1.

5.3.2 Preliminary validation of Ov2 as a target of OvHV-2-encoded miRNAs by flow cytometry

The coding region of Ov2 was cloned into pEGFPN1 (construct made by Dr Inga Dry). Transfections and flow cytometry were carried out as described in sections 2.1.7, 2.6.2 and 2.6.4 with n = 3 for each condition. Between replicates and conditions the percentage of GFP positive cells remained consistent (on average 16.26%, standard deviation 2.71) indicating the transfection efficiency was not altered when the EGFPN1 constructs were co-transfected with different mimics. GFP positive cells were counted and the median fluorescent intensity (MFI) for each replicate was determined and used as an indicator of GFP expression levels (Fig. 5.2). Statistically significant reductions in the MFI were observed when ovhv2-miR-17-10 ($p \leq 0.01$), ovhv2-miR-61-1 ($p \leq 0.0001$) and ovhv2-miR-73-1 ($p \leq 0.05$) were co-transfected with an Ov2-EGFPN1 construct compared to the scrambled siRNA. A significant increase in the MFI was observed when ovhv2-miR-17-29 ($p \leq 0.05$) was co-transfected with an Ov2-EGFPN1 construct compared to the scrambled siRNA. When all four of the OvHV-2-encoded miRNAs predicted to target Ov2 were co-transfected with an Ov2-EGFPN1 construct the reduction in MFI observed compared to the scrambled siRNA was not significant. From this preliminary experiment, ovhv2-miR-17-10 and ovhv2-miR-61-1 were taken forward for further validation.

```
MIRNA  : ovhv2-miR-17-29
length: 23

mfe: -18.2 kcal/mol
p-value: undefined

position  46
target 5'   C    AA   AAACAUA       A 3'
             CUGA  AGA       UCCAAGA
             GACU  UCU       AGGUUCU
miRNA  3' GAU    G    ACGC          A 5'

MIRNA  : ovhv2-miR-17-10
length: 23

mfe: -23.8 kcal/mol
p-value: undefined

position  104
target 5'   G    A  ACAAAUAGAAG   AUCAC         A 3'
             CAGG GC           AGC     GUAACUUCA
             GUCC CG           UCG     CAUUGAAGU
miRNA  3' UAG    A               A              5'

MIRNA  : ovhv2-miR-61-1
length: 25

mfe: -30.7 kcal/mol
p-value: undefined

position  454
target 5'      C    AA      UUAUG       C 3'
                UCAG  CAGCAC     GUCCCCA
                AGUC  GUCGUG     CAGGGGU
miRNA  3' UGCAGC    G                   U 5'


MIRNA  : ovhv2-miR-73-1
length: 23

mfe: -22.0 kcal/mol
p-value: undefined

position  304
target 5'  C      GG    A UGAAAAGAAAAAAGA        G 3'
            UUGCGG  GGAG U               AGAGAUUA
            AAUGUU  CCUC G               UCUCUAAU
miRNA  3' UA      AA                             5'
```

Figure 5.1 RNAHybrid analysis of OvHV-2-encoded miRNAs against Ov2

mfe = minimum free energy. Constraints on miRNA binding included perfect complementarity between nucleotides 2 and 8 and no G:U pairing within this region. Positions refer to nucleotide start sites in the Ov2 gene (AY839756.1, reverse complement of nucleotides 1321 – 2480). Targets shown are located in the coding region.

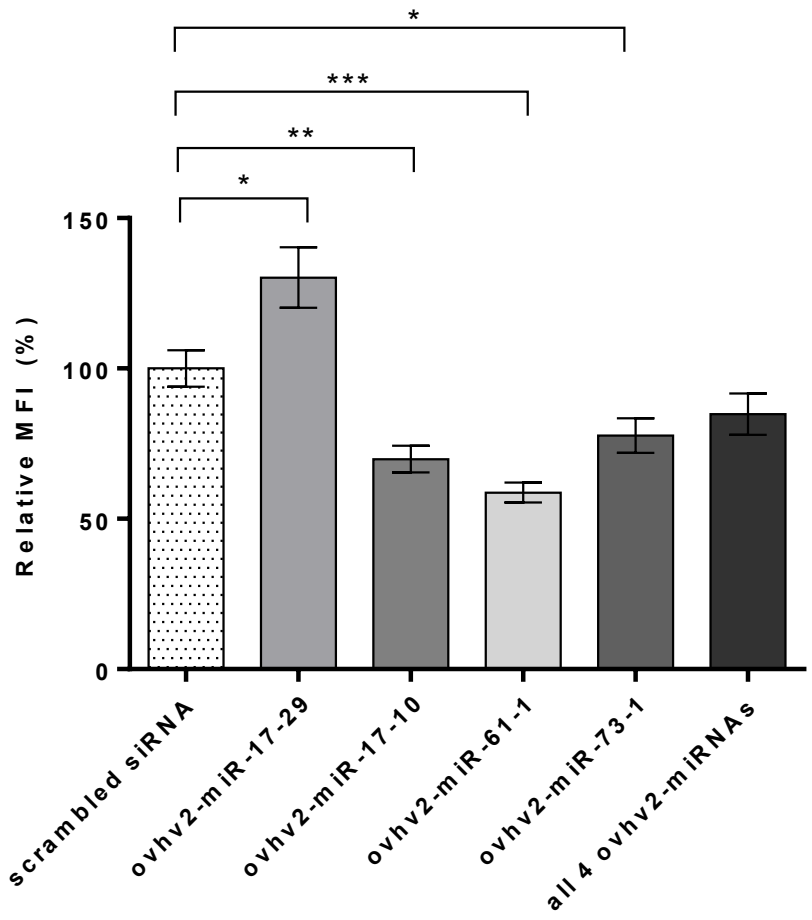


Figure 5.2 Relative MFI of OV2-EGFPN1 with OvHV-2-encoded miRNAs compared to a scrambled siRNA control

293T cells were co-transfected with an Ov2-EGFPN1 construct and OvHV-2-encoded miRNAs predicted to target the coding region of Ov2 or a scrambled siRNA. The MFI for each sample was determined 48 hrs later by flow cytometry and test ovhv2-miRNAs were compared to a scrambled siRNA control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.0001$.

5.3.3 Validation of Ov2 as a target of ovhv2-miR-17-10 and ovhv2-miR-61-1 by flow cytometry

Transfections and flow cytometry were carried out as described in sections 2.1.7, 2.6.2 and 2.6.4 with n = 14 for each condition. Between replicates and conditions the percentage of GFP positive cells remained consistent (on average 31.5%, standard deviation 4.84) indicating the transfection efficiency was not altered when the EGFPN1 constructs were co-transfected with different mimics. GFP positive cells were counted and the MFI for each replicate was determined and used as an indicator of GFP expression levels (Fig. 5.3). Significant decreases ($p \leq 0.0001$ for all) of 35%, 39% and 44% in the MFI were observed when ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both ovhv2-miRs were cotransfected with an Ov2-EGFPN1 construct respectively compared to a scrambled siRNA. No significant changes in the MFI were observed when ovhv2-miR-17-10 or a combination of both ovhv2-miRs were cotransfected with an EGFPN1 construct compared to a scramble siRNA. A significant decrease of 25% ($p \leq 0.01$) in the MFI was observed when ovhv2-miR-61-1 was co-transfected with an EGFPN1 construct compared to the scrambled siRNA.

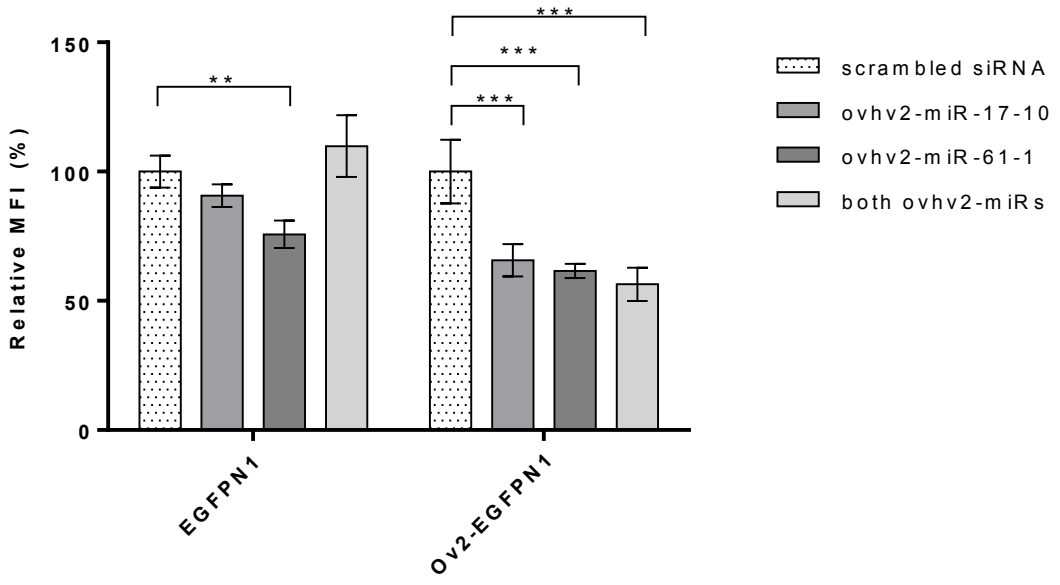


Figure 5.3 Relative MFI of EGFPN1 and Ov2-EGFPN1 with ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both compared to a scrambled siRNA

293T cells were co-transfected with either an EGFPN1 or an Ov2-EGFPN1 construct and ovhv2-miR-17-10, ovhv2-miR-61-1, a combination of both ovhv2-miRs or a scrambled siRNA. The MFI for each sample was determined 48 hrs later by flow cytometry and test ovhv2-miRNAs were compared to a scrambled siRNA control. ** = $p \leq 0.01$, *** = $p \leq 0.0001$.

5.3.4 Validation of Ov2 as a target of ovhv2-miR-17-10 and ovhv2-miR-61-1 by western blotting

The coding region of Ov2 was cloned into pcDNA3.1+ (construct made by Dr Inga Dry). Transfections of cells seeded in 12 well plates were carried out as described in section 2.1.7. Western Blotting was performed as described in section 2.4. Membranes were incubated with a rabbit anti-Ov2 and a mouse anti-actin primary antibodies before incubation with appropriate secondary antibodies. One representative western blot is shown in Fig. 5.4. A total of three independent western blots were performed with duplicates of each condition (Ov2-pcDNA3.1+ co-transfected with one of scrambled siRNA, ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both) on each western blot (n = 6 total for each condition). For each sample, the levels of Ov2 were quantified and normalised to the level of actin. Test ovhv2-miRNA samples were then compared to the scrambled siRNA (Fig. 5.6). When either ovhv2-miR-17-10 or ovhv2-miR-61-1 were co-transfected with an Ov2-pcDNA3.1+ construct statistically significant reductions of approximately 40% ($p \leq 0.05$ for both ovhv2-miRNAs) were observed compared to the scrambled siRNA. When both ovhv2-miRNAs were co-transfected with an Ov2-pcDNA3.1+ construct a significant reduction ($p \leq 0.01$) of approximately 60% was observed compared to the scrambled siRNA.

5.3.5 Mutagenesis of the ovhv2-miR-17-10 and ovhv2-miR-61-1 Ov2 binding sites

The binding sites of ovhv2-miR-17-10 and ovhv2-miR-61-1 (as shown in Fig 5.1) were mutated by site-directed mutagenesis (as described in section 2.2.8). The coding sequence of Ov2 was not altered. Western blotting was carried out with the same conditions as described in section 5.3.4. One representative western blot for each mutated Ov2-pcDNA3.1+ construct is shown in Fig. 5.5. A total of two independent western blots were performed with duplicates of each condition on each blot (n = 4 for each condition). For each sample, the levels of Ov2 were quantified and normalised to the level of actin. Test ovhv2-miRNA samples were then compared to the scrambled siRNA (Fig. 5.6). When ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both ovhv2-miRNAs were co-transfected with an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-17-10 binding site significant reductions of approximately 55%, 60% and 70% respectively ($p \leq 0.01$ for all conditions) were observed compared to the scrambled siRNA. When ovhv2-miR-17-10 was co-transfected with an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-61-1 binding site a significant reduction ($p \leq 0.05$) of approximately 40% was observed compared to the scrambled siRNA. When ovhv2-miR-61-1 was co-transfected with an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-61-1 binding site no significant reduction was observed compared to the scrambled siRNA. When

a combination of both ovhv2-miRNAs were co-transfected with an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-61-1 binding site a significant reduction ($p \leq 0.01$) of approximately 50% was observed compared to the scrambled siRNA. These results, especially the continued inhibition observed in the Ov2 construct with a mutated ovhv2-miR-17-10 binding site, will be discussed at the end of this chapter.

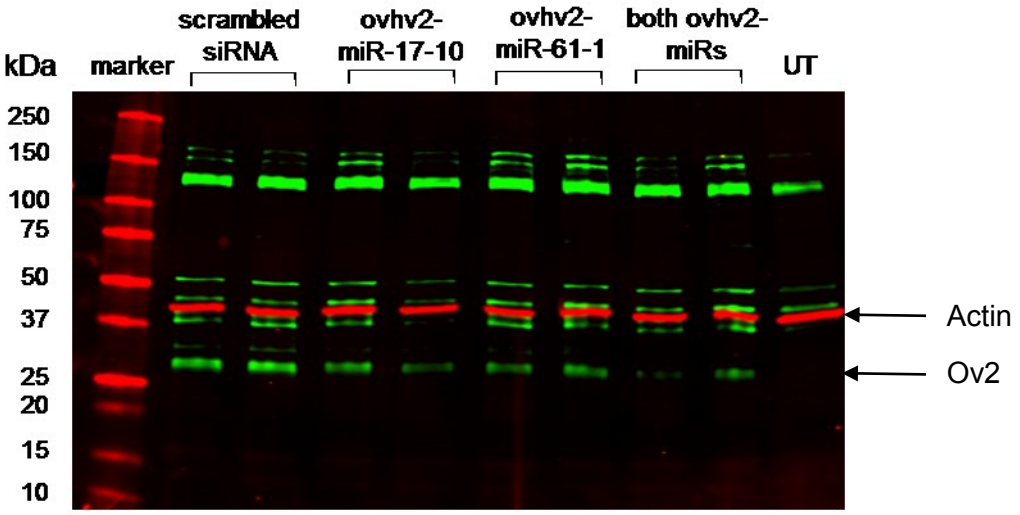


Figure 5.4 Western Blot of Ov2-pcDNA3.1+ with ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both compared to a scrambled siRNA

293T cells were co-transfected with an Ov2-pcDNA3.1+ construct and ovhv2-miR-17-10, ovhv2-miR-61-1, a combination of both ovhv2-miRs or a scrambled siRNA. Cells were harvested 48 hrs post transfection and western blotting performed. UT = untransfected control. One representative western blot is shown.

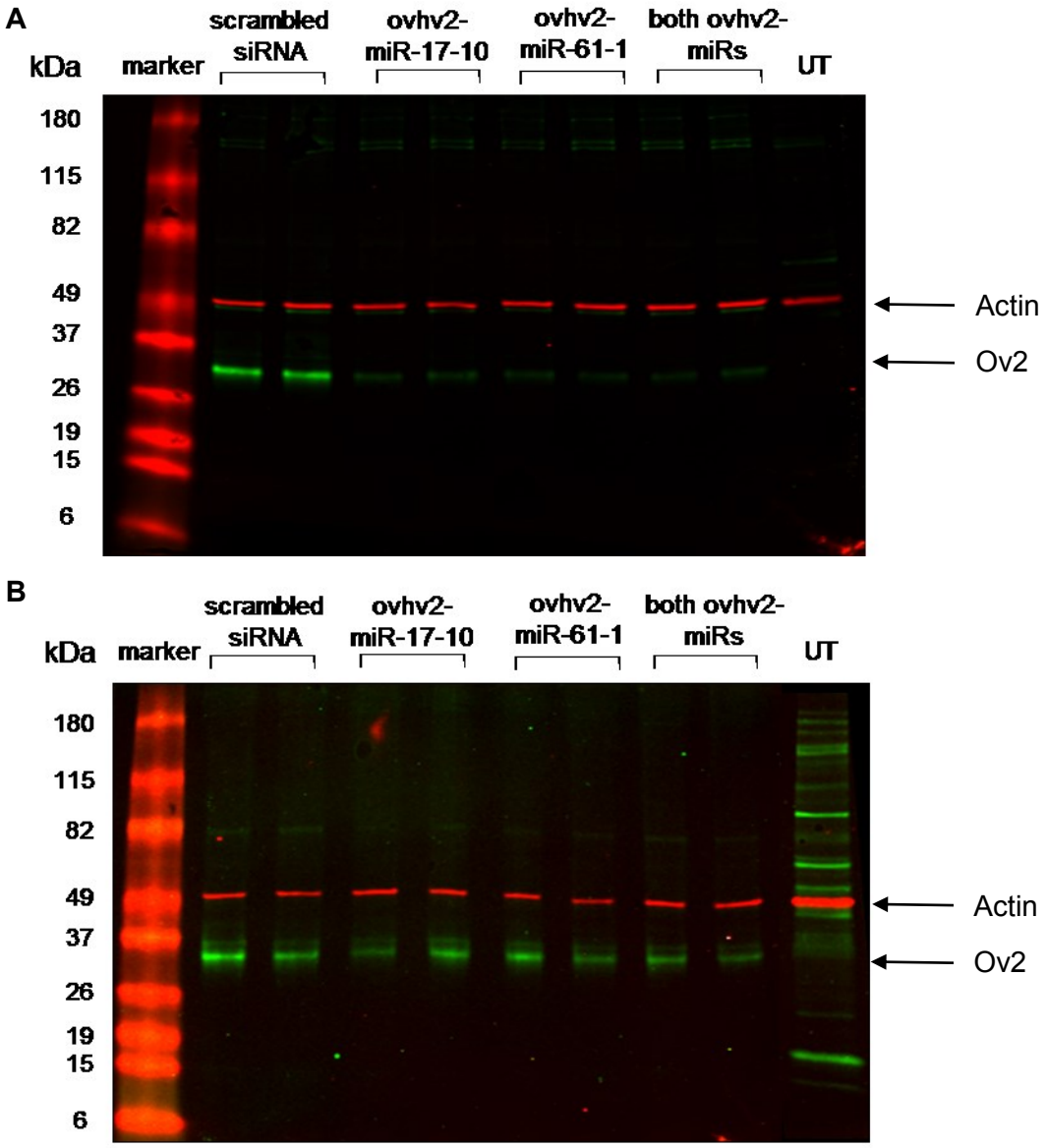


Figure 5.5 Western Blots of Ov2-pcDNA3.1+ with mutated ovhv2-miR-17-10 or ovhv2-miR-61-1 binding sites with ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both compared to a scrambled siRNA

293T cells were co-transfected with either an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-17-10 binding site (A) or an Ov2-pcDNA3.1+ construct with a mutated ovhv2-miR-61-1 binding site (B) and ovhv2-miR-17-10, ovhv2-miR-61-1, a combination of both ovhv2-miRs or a scrambled siRNA. Cells were harvested 48 hrs post transfection and western blotting performed. UT = untransfected control. One representative western blot is shown for each Ov2 construct.

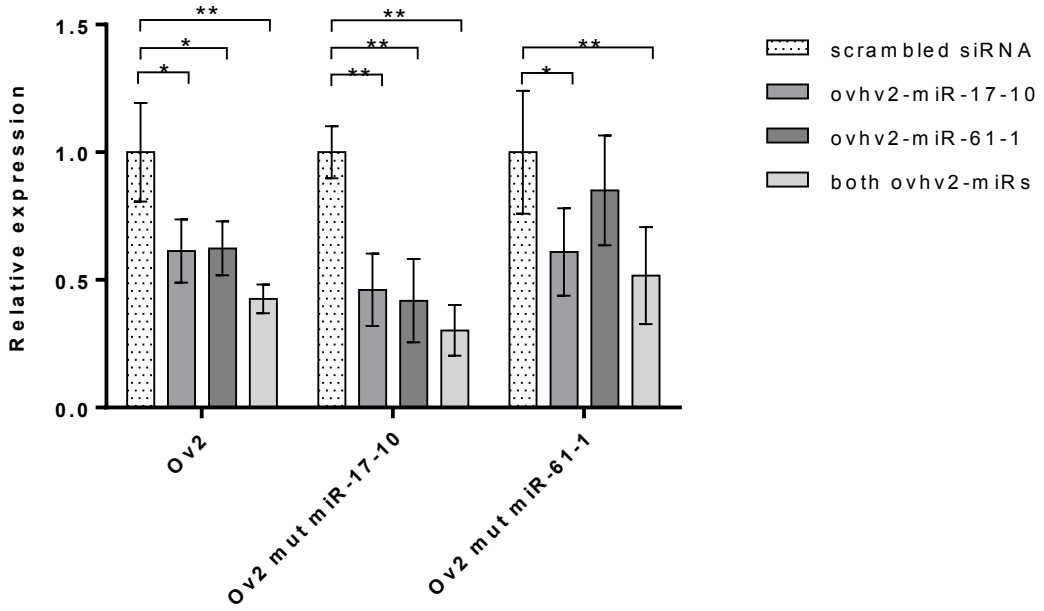


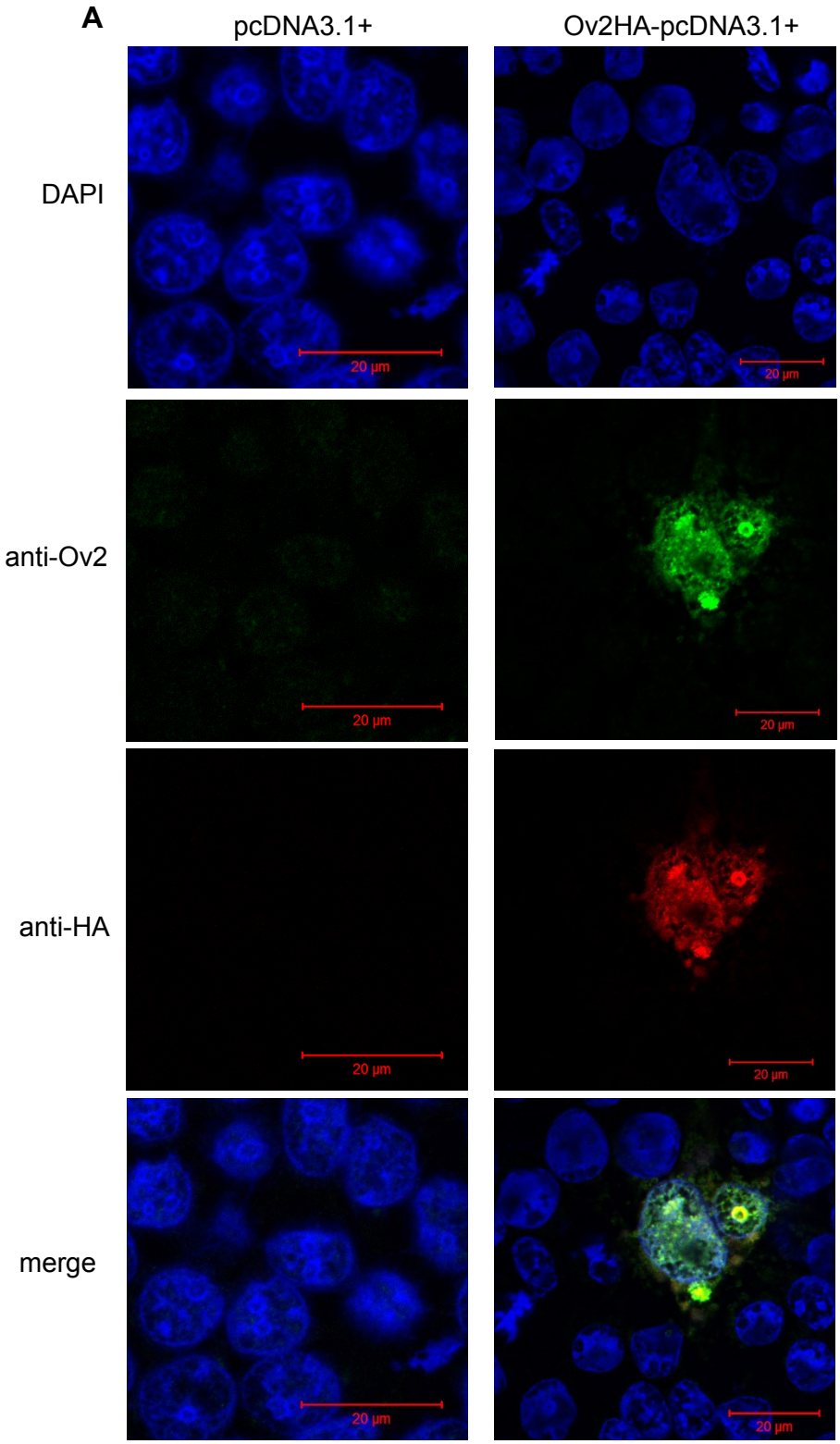
Figure 5.6 Relative expression of Ov2-pcDNA3.1+ and mutated Ov2-pcDNA3.1+ constructs with ovhv2-mIR-17-10, ovhv2-miR-61-1 or a combination of both compared to a scrambled siRNA

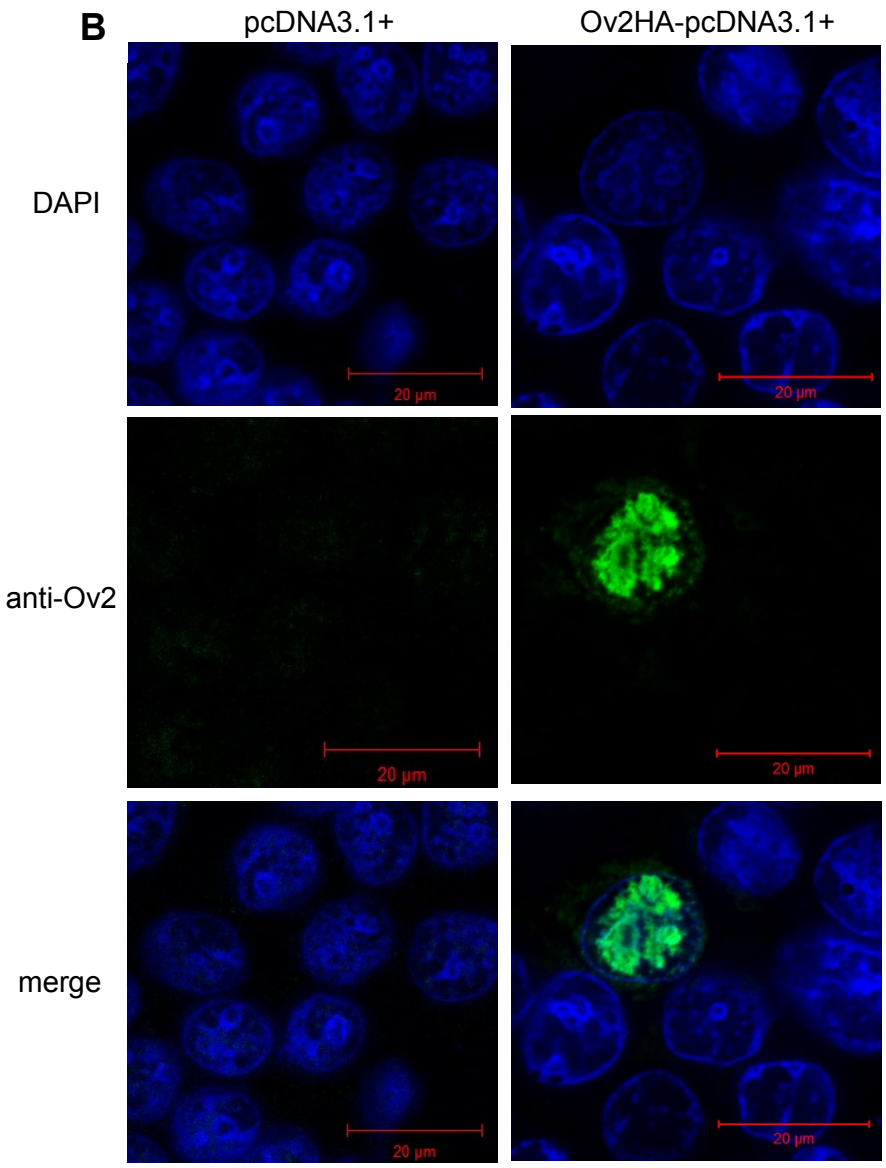
Western blots were quantified using the LI-COR system. Expression of Ov2 was normalised to expression of actin for each sample. Test ovhv2-miRs were then compared to a scrambled siRNA. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.0001$.

5.4 Functional Analysis of Ov2

5.4.1 Determining the subcellular localisation of Ov2

Coverslips were prepared and transfected with either an Ov2HA-pcDNA3.1+ construct or a pcDNA3.1+ construct as described in section 2.1.8. 24 hrs. Fixing and immunofluorescence was carried out 24 hrs post transfection as described in section 2.7.1. Coverslips were incubated with a rabbit anti-Ov2 primary antibody or a rat anti-HA primary antibody or both primary antibodies before incubation with appropriate secondary antibodies. The nucleus was stained for using DAPI in all coverslips. Confocal microscopy was performed as described in section 2.7.2. Representative images for each condition can be seen in Figure 5.7. Good co-localisation of the anti-Ov2 and anti-HA antibody was observed (Fig 5.7. C) and Ov2 appears to have a nuclear localisation. To ensure the localisation observed was not due to overexpression and accumulation of Ov2 protein at 24 hrs further coverslips were prepared and transfected with either an Ov2HA-pcDNA3.1+ construct or a pcDNA3.1+ construct and stained for Ov2 with the anti-Ov2 antibody at 4 hrs, 8 hrs and 16 hrs post transfection. Figure 5.8 shows representative images for each of these times points and even from the earliest time point of 4 hrs post transfection Ov2 is observed to be located in the nucleus.


A
pcDNA3.1+
Ov2HA-pcDNA3.1+
DAPI
anti-Ov2
anti-HA
merge
20 μm


B
pcDNA3.1+
Ov2HA-pcDNA3.1+
DAPI
anti-Ov2
merge
20 μm

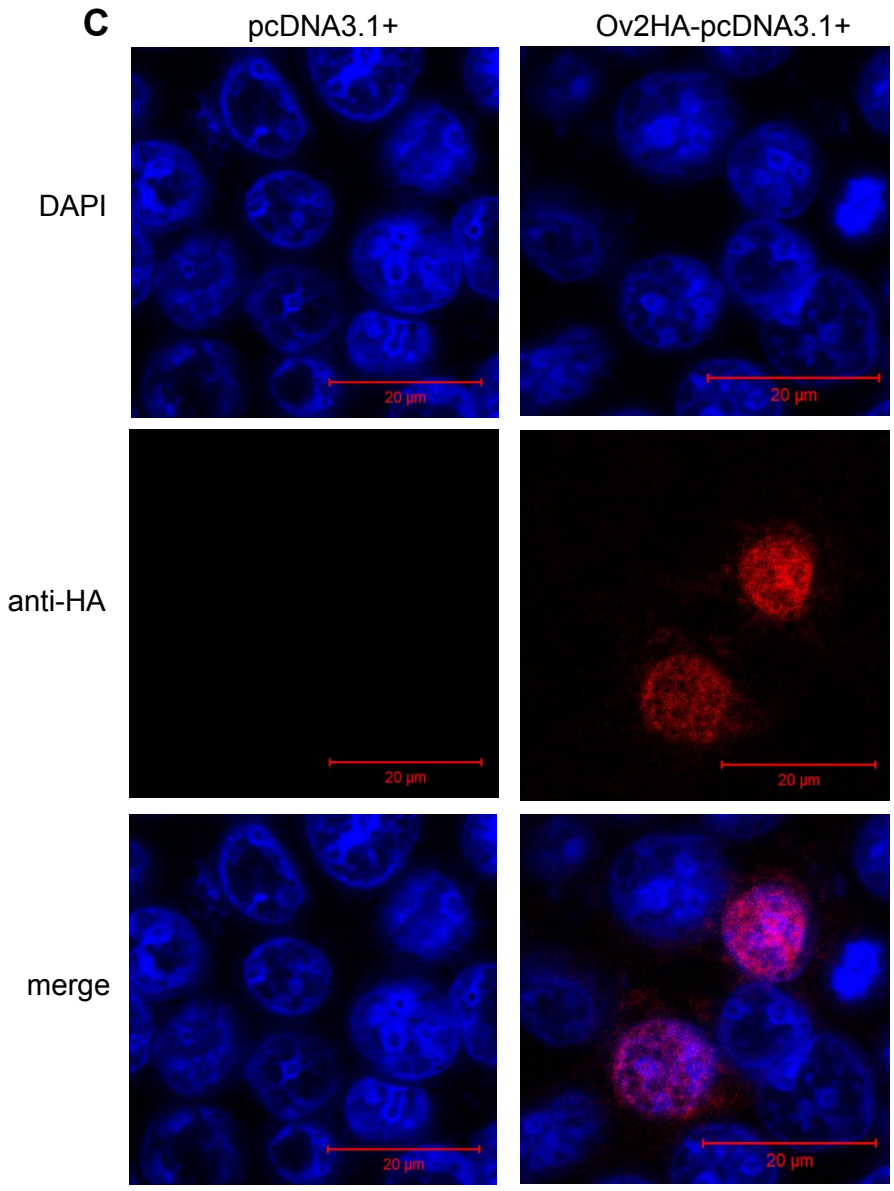


Fig 5.7 Immunofluorescence of pcDNA3.1+ or Ov2HA-pcDNA3.1+ stained with either an anti-HA antibody, an anti-Ov2 antibody or a combination of both at 24 hrs post transfection

293T cells were either transfected with a pcDNA3.1+ construct or an Ov2HA-pcDNA3.1+ construct. Fixing and immunostaining of cells was performed 24 hrs post transfection. Cells were either stained with a combination of an anti-Ov2 antibody and an anti-HA antibody (panels in A), an anti-Ov2 antibody (panels in B) or an anti-HA antibody (panels in C). DAPI staining was performed in all conditions to stain the nucleus.

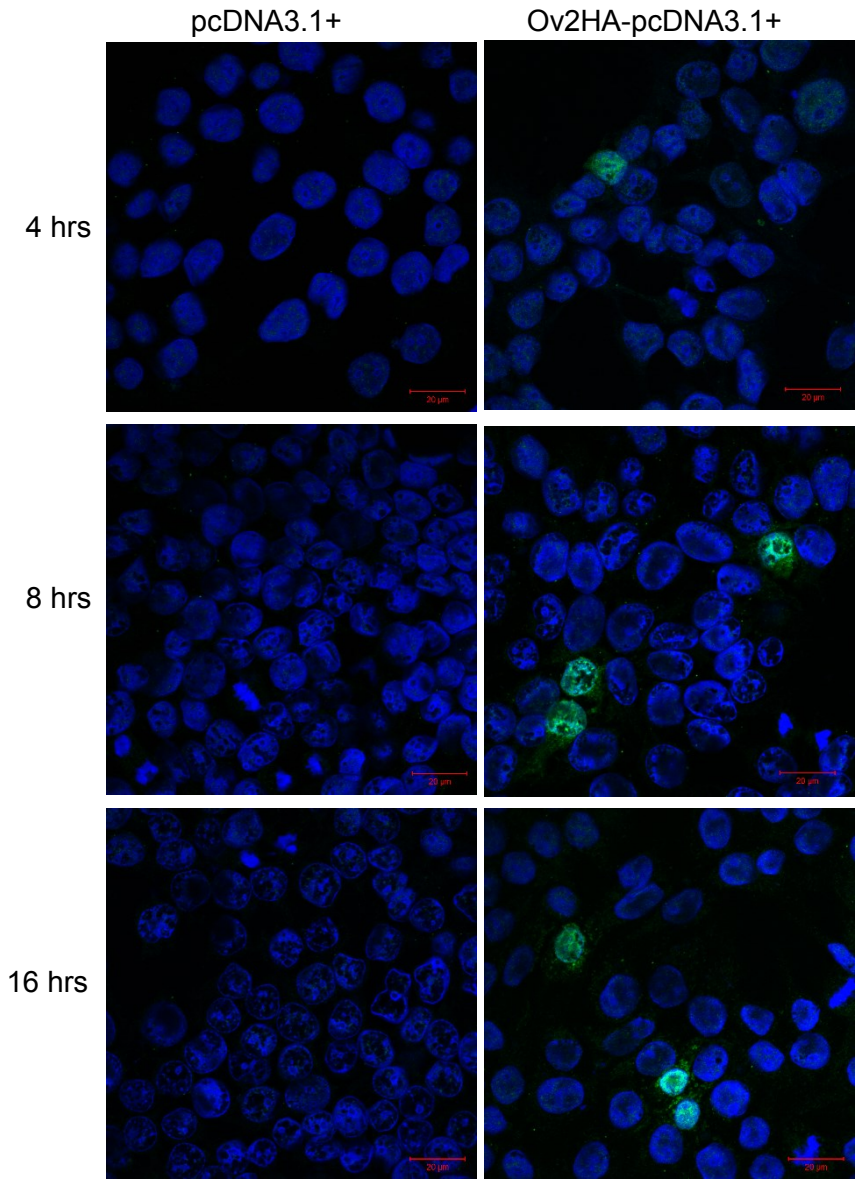


Fig 5.8 Immunofluorescence of pcDNA3.1+ or Ov2HA-pcDNA3.1+ stained with an anti-Ov2 antibody at 4 hrs, 8 hrs and 16 hrs post transfection

293T cells were either transfected with a pcDNA3.1+ construct or an Ov2HA-pcDNA3.1+ construct. Fixing and immunostaining of cells was performed 4 hrs, 8 hrs and 16 hrs post transfection. Cells were stained with an anti-Ov2 antibody (green) and DAPI (blue). Merged images are shown above. Split images can be found in Appendix 6.

5.4.2 Regulation of Jagged and Caspase 8 by Ov2

In the study by Parameswaran *et al.* a number of genes were identified by RNA-Seq that were differentially regulated by an A2 deletant virus compared to wild type or a revertant virus. Two of these genes, *JAG1* (Jagged) and *CASP8*, were investigated to see whether they were differentially regulated by Ov2. MDBK cells were nucleofected with either an *Ov2*-EGFPN1 construct or an EGFPN1 construct as described in section 2.1.9. cDNA was made from RNA harvested 48 hrs post nucleofection as described in sections 2.2.2 and 2.2.3. RT-qPCR for four biological replicates (with two technical replicates per sample) were carried out as described in section 2.2.10 for *JAG1* and *CASP8* (Figure 5.9). Expression of both *JAG1* and *CASP8* were normalised to the expression of *GAPDH* for each sample and the ΔΔCt method of analysis was used to determine fold change for Ov2-EGFPN1 nucleofected cells compared to EGFPN1 transfected cells (Livak & Schmittgen, 2001). No significant changes were observed in the relative expression levels of *CASP8* when an Ov2-EGFPN1 construct was nucleofected compared to an EGFPN1 construct. A statistically significant reduction of approximately 25% ($p \leq 0.001$) was observed in the relative expression levels of *JAG1* when an Ov2-EGFPN1 construct was nucleofected compared to an EGFPN1 construct.

5.4.3 Ov2 immunoprecipitation and Mass Spectrometry analysis

To determine potential interacting partners of Ov2, immunoprecipitation and subsequent mass spectrometry analysis was performed. 293T cells were either transfected with a pcDNA3.1+ construct or an Ov2HA-pcDNA3.1+ construct as described in section 2.1.7. Cell lysates were harvested 48 hrs post transfection as described in section 2.5.1. Immunoprecipitation using HA-tagged magnetic beads was carried out in duplicate as described in section 2.5.2. Western blotting was performed (as described in sections 2.4.2 to 2.4.4) on one set of samples to confirm the successful immunoprecipitation of Ov2 (Figure 5.10). Ov2 protein can be detected by the anti-Ov2 antibody in the Ov2HA-pcDNA3.1+ input and eluted samples but not in the pcDNA3.1+ input or eluted samples confirming that the immunoprecipitation was a success. The other set of samples immunoprecipitated at the same time were sent for mass spectrometry analysis as described in section 2.5.3. Analysis of the mass spectrometry data was performed using the following constraints: proteins must have a protein score of over forty and there must be more than one unique peptide matching the protein. The proteins unique to either pcDNA3.1+ or Ov2HA-pcDNA3.1+ are listed in Table 5.1A and 5.1B respectively. A large number of proteins were present in both pcDNA3.1+ and Ov2HA-pcDNA3.1+ samples; proteins that had a two-fold higher protein score in the Ov2HA-pcDNA3.1+ samples compared to the pcDNA3.1+ samples are listed in Table 5.2. For reference, the full list of proteins that met the constraints for both samples can be found in Appendix 7.

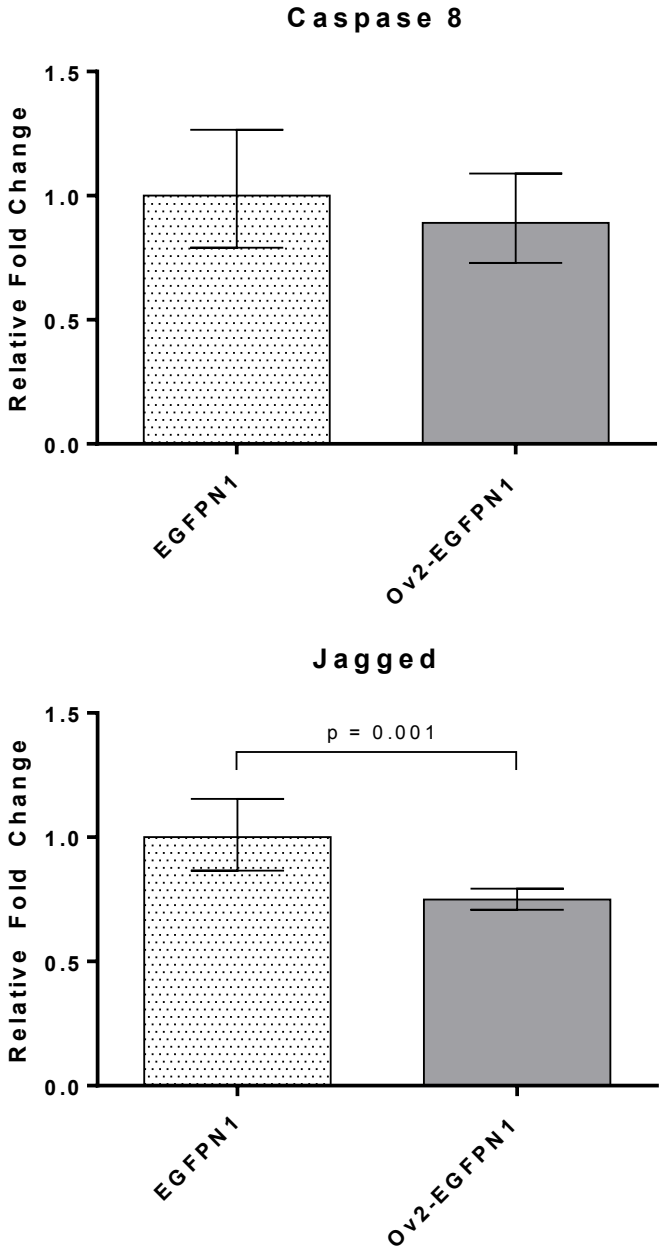


Figure 5.9 Relative expression of *CASP8* and *JAG1* in Ov2-*EGFPN1* transfected cells compared to *EGFPN1* transfected cells

MDBK cells were nucleofected with either an Ov2-*EGFPN1* construct or an EGFPN1 construct. cDNA was made from RNA harvested 48 hrs post nucleofection and RT-qPCR performed. The ΔΔCt method of analysis was used to determine fold change of Ov2-*EGFPN1* transfected cells compared to EGFPN1 transfected cells. The expression of *CASP8* and *JAG1* was normalised to the expression of *GAPDH* for each sample. Error bars represent upper and lower limits.

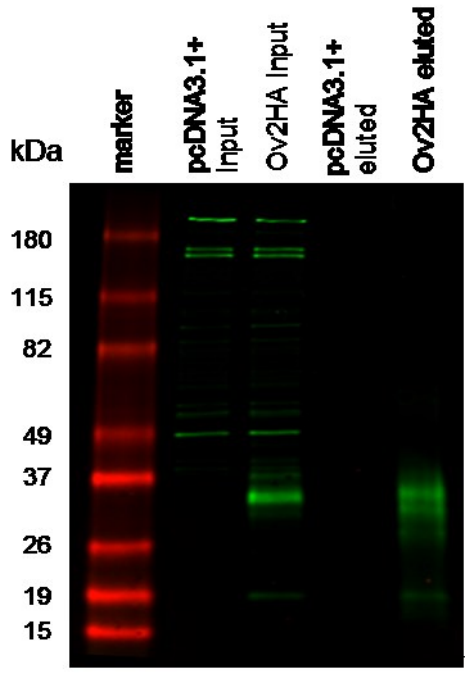


Figure 5.10 Western Blotting of pcDNA3.1+ and Ov2HA-pcDNA3.1+ immunoprecipitated samples

293T cells were either transfected with a pcDNA3.1+ construct or an Ov2HA-pcDNA3.1+ construct. Cell lysates were harvested 48 hrs post transfection and immunoprecipitated using HA-tagged magnetic beads. Western blotting was performed to confirm the presence of Ov2 in the immunoprecipitated samples.

Table 5.1 Unique proteins identified by Mass Spectrometry in pcDNA3.1+ and Ov2HA-pcDNA3.1+ immunoprecipitated samples

A: pcDNA3.1+ sample

Protein ID	Gene Name	Protein Score	No. Unique Peptides
Q5VTE0	Putative elongation factor 1-alpha-like 3 (EEF1A1P5)	180	5
P58876	Histone H2B type 1-D (HIST1H2BD)	80	3
H0YMV8	40S ribosomal protein S27 (RPS27L)	52	2
K7EMH1	60S ribosomal protein L22 (Fragment) (RPL22)	46	2

B: Ov2HA-pcDNA3.1+ sample

Protein ID	Gene Name	Protein Score	No. Unique Peptides
Q9NQ29	Putative RNA-binding protein Luc7-like 1 (LUC7L)	254	4
P46776	60S ribosomal protein L27a (RPL27A)	199	5
P42766	60S ribosomal protein L35 (RPL35)	199	2
P08238	Heat shock protein HSP 90-beta (HSP90AB1)	142	4
P07195	L-lactate dehydrogenase B chain (LDHB)	121	2
H0Y5B4	60S ribosomal protein L36a (Fragment) (RPL36A)	115	2
E9PI65	Heat shock cognate 71 kDa protein (Fragment) (HSPA8)	106	3
F5H4F9	40S ribosomal protein S3a (RPS3A)	99	3
J3KPD9	Nucleoside diphosphate kinase B (NME2)	74	2
P50990	T-complex protein 1 subunit theta (CCT8)	62	2
P62241	40S ribosomal protein S8 (RPS8)	60	2
Q2VSN7	OvHV-2 Ov2 protein	55	2

Table 5.2: Proteins identified by Mass Spectrometry that have a Protein Score that is at least 2 Fold higher in Ov2HA-pcDNA3.1+ samples compared to pcDNA3.1+ samples

Protein ID	Gene Name	pcDNA3.1+ Protein Score	Ov2HA-pcDNA3.1+ Protein Score	Fold Difference
Q06830	Peroxiredoxin-1 (PRDX1)	47	185	3.94
P62854	40S ribosomal protein S26 (RPS26)	74	287	3.88
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	52	181	3.48
Q9Y383	Putative RNA-binding protein Luc7-like 2 (LUC7L2)	223	593	2.66
P39019	40S ribosomal protein S19 (RPS19)	157	371	2.36
P06733	Alpha-enolase (ENO1)	134	315	2.35
P10809	60 kDa heat shock protein, mitochondrial (HSPD1)	139	316	2.27
E9PR30	40S ribosomal protein S30 (FAU)	104	234	2.25
P14618	Pyruvate kinase PKM (PKM)	93	206	2.22
P62701	40S ribosomal protein S4, X isoform (RPS4X)	356	757	2.13

5.5 Discussion

It was demonstrated that Ov2 is a target of ovhv2-miR-17-10 and ovhv2-miR-61-1. RNAHybrid was initially used to probe the Ov2 sequence for target sites of OvHV-2-encoded miRNAs. In total, eight sites were predicted (four in the coding region, see section 5.3.1 and four in the 3' UTR, see Appendix 9). As the coding region of Ov2 has already been cloned into a number of vectors (pcDNA3.1+, pcDNA3.1+ with an HA tag and pEGFPN1) only the coding region sites were investigated. It is worth noting that ovhv2-miR-17-13 had two predicted target sites in the 3'UTR that were in close proximity of each other. It is thought that when two binding sites for the same miRNA are present the magnitude of regulation is increased (Brennecke *et al.*, 2005; Sætrom *et al.*, 2007). If this work was to be continued it would therefore be of interest to investigate whether any of the predicted 3'UTR target sites are functional.

It was traditionally thought that functional sites of miRNAs only existed in the 3'UTR however there is increasing evidence that miRNAs can bind to sites in the coding region with functional activity (Hausser *et al.*, 2013). Moreover, it has been shown that small interfering RNAs that target the coding region can be effective at silencing (Saxena *et al.*, 2003). In fact, there are a number of cases of targeting in the coding region by miRNAs in the literature, including a coding region target for the *let-7* miRNA within Dicer, part of the miRNA biogenesis machinery (Elcheva *et al.*, 2009; Forman *et al.*, 2008; Liu *et al.*, 2015a; Qin *et al.*, 2010; Tay *et al.*, 2008). However, it should be noted that it is thought that targeting in the coding region is often less effective in down-regulation of target transcript levels but can result in a similar efficiency of translational knockdown as targeting of the 3'UTR (Baek *et al.*, 2008; Fang & Rajewsky, 2011; Guo *et al.*, 2010).

A preliminary screen of the four predicted coding region target sites was carried out using an Ov2-EGFPN1 construct. It had originally been planned to use a plate reader to measure the GFP signal as this would have been a very quick and simple method. However, the GFP signal was significantly reduced in the Ov2-EGFPN1 construct and the plate reader was no longer a useable method of quantifying GFP signal. Instead, flow cytometry was used to measure the intensity of the GFP signal and also had the added benefit of ensuring similar numbers of cells were transfected between samples. One miRNA, ovhv2-miR-17-29, appeared to increase the MFI of an Ov2-EGFPN1 compared to a scrambled siRNA control. There are a number of reports documented in which a miRNA appears to have a positive effect on a target transcript (Andersson Ørom *et al.*, 2008; Ghosh *et al.*, 2008; Lu *et al.*, 2010b; Ma *et al.*, 2010; Vasudevan *et al.*, 2007). For the purposes of this thesis, the effect of ovhv2-miR-17-29 on Ov2 was not characterised any further. The other three miRNAs, ovhv2-miR-17-10, ovhv2-miR-61-1 and ovhv2-miR-73-1 all showed significant decreases in the MFI of an Ov2-EGFPN1 construct compared to a scrambled siRNA control. The two that displayed the strongest effect on the MFI, ovhv2-miR-17-10 and ovhv2-miR-61-1 were taken forward for further validation.

The same method of validation was used as in the preliminary screen but this time the number of biological replicates was increased to 14 and appropriate controls were used. An empty vector control was included to check for off-target effects of ovhv2-miR-17-10 and ovhv2-miR-61-1. Similar levels of knockdown were observed for the miRNAs as in the preliminary screen however there was a statistically significant knockdown when ovhv2-miR-61-1 was co-transfected with pEGFPN1 compared to a scrambled siRNA. The reduction observed was not as high as when ovhv2-miR-61-1 was co-transfected with Ov2-EGFPN1 however another method of validation, not using EGFPN1 was required to confirm Ov2 as a target of ovhv2-miR-61-1. RNAHybrid analysis of ovhv2-miR-61-1 against the pEGFPN1 vector revealed two potential target sites in the coding region of EGFP; both of which had perfect complementarity between nucleotides 1 and 7 and used G:U pairing in this region and so it is therefore possible that there are off-target effects of ovhv2-miR-61-1 on the vector which may potentially explain the knockdown observed in the pEGFPN1 control samples.

As an antibody to Ov2 was available western blotting was performed on an untagged Ov2-pcDNA3.1+ construct co-transfected with a scrambled siRNA, ovhv2-miR-17-10, ovhv2-miR-61-1 or a combination of both ovhv2-miRNAs. The levels of knockdown of Ov2 were similar to that observed by flow cytometry indicating that ovhv2-miR-61-1 does target Ov2. To confirm that the sites predicted by RNAHybrid were the actual binding sites of ovhv2-miR-17-10 and ovhv2-miR-61-1 site directed mutagenesis was performed, whilst keeping the coding sequence the same. For ovhv2-miR-61-1, the complete binding site was mutated however for ovhv2-miR-17-10 only the seed sequence binding site was mutated due to the length of the predicted binding site. When ovhv2-miR-61-1 was co-transfected with the Ov2-pcDNA3.1+ construct with the relevant predicted binding site mutated no knockdown of Ov2 levels were observed. It is thus likely that the predicted target site for ovhv2-miR-61-1 is the actual binding site and results in functional knockdown of Ov2 protein levels. This mutation did not affect the knockdown of Ov2 by ovhv2-miR-17-10. The levels of knockdown of Ov2 when both ovhv2-miRNAs were co-transfected with an Ov2-pcDNA3.1+ construct were also not affected, indicating that there is no combinatorial effect of the two ovhv2-miRNAs.

When ovhv2-miR-17-10 was co-transfected with the Ov2-pcDNA3.1+ construct with the relevant predicted binding site mutated knockdown of Ov2 was observed to similar levels as in the wild-type Ov2-pcDNA3.1+ construct. To disrupt ovhv2-miR-17-10 binding, three amino acids (an arginine, an asparagine and a phenylalanine) were mutated. There are only two codons available for asparagine (AAC and AAT) and phenylalanine (TTC and TTT) and as such mutation of these two amino acids results in changes in the base pairing of ovhv2-miR-17-10 with Ov2 from G:C to G:U. G:U pairing is allowed in seed sequences and can still result in functional knockdown of the target and as such this could explain why there was no restoration of Ov2 levels observed in the mutated construct. Furthermore, there are another two sites present in the Ov2 coding region that allow for G:U pairing with perfect complementarity between either nucleotides 1 and 7 or 2 and 8 (see Appendix 10 for

RNAHybrid analyses). Therefore from this analysis it cannot be confirmed whether the initial predicted ovhv2-miR-17-10 target site is the actual binding site and further investigation would be required, such as changing the protein sequence of Ov2 or using a different validation method. However, changing the coding sequence of Ov2 may have other unknown consequences on the activity of Ov2. Using a different approach such as the luciferase assays used for validation of *DLL1* and MHC class II genes is beneficial as the sequence cloned into the vector can be mutated freely as the multiple cloning site is located in the 3'UTR of renilla (see appendix 1 for vector map of psiCHECK-2). However, there are limitations of the luciferase assay approach which are discussed in further details in chapter 6.

The second part of this thesis chapter investigated the functional activity of Ov2 in a number of ways. Firstly, the subcellular localisation of Ov2 was determined and it was found to be localised in the nucleus. Importantly, there was a good correlation between the anti-HA antibody and anti-Ov2 antibody used, and an untagged Ov2 construct had the same subcellular localisation as the HA-tagged Ov2 construct (see Appendix 6). The subcellular localisation of Ov2 observed is unlikely to be an artefact of overexpression of the construct as the same distribution of Ov2 was seen at 4 hrs, 8 hrs and 16 hrs post transfection. The nuclear localisation of Ov2 is not surprising as the sequence has two predicted nuclear localisation signal sequences, and as Ov2 is predicted to contain a bZIP domain it is likely to have a role in transcription.

A second approach to investigating the function of Ov2 was based on the work published by Parameswaran *et al.* on the A2 protein of AlHV-1. A number of genes identified as either being up-regulated or down-regulated during infection of rabbits with an A2 deletant virus compared to a wild-type or revertant virus were selected for RT-qPCR analysis. Genes selected for analysis that were up-regulated during infection with an A2 deletant virus included *CASP8*, DENN/MADD Domain Containing 2D (*DENND2D*, a guanine nucleotide exchange factor), Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1 (*NFKB1*, a transcriptional regulator), Cyclin-Dependent Kinase 2 (*CDK2*, involved in cell cycle regulation) and *IL-2*. Genes selected for analysis that were down-regulated during infection with an A2 deletant virus included *JAG1*, *PRF1* (perforin, a component of cytolytic granules), Nuclear Factor Of Activated T-Cells, Cytoplasmic, Calcineurin-Dependent 2 (*NFATC2*, a transcription factor that plays a central role during activation of the immune response), *GZMA* (another component of cytolytic granules) and *FOSB*. Due to limited availability of relevant cell lines not all of these genes were expressed in the MDBK cell line chosen; as expected the genes with expression restricted to lymphocytes such as *PRF1*, *IL2*, *NFATC2* and *GZMA* were not expressed in MDBK cells. Furthermore, due to time restrictions, primer sets could not be optimised for *NFKB1*, *CDK2* and *DENND2D*. Finally, although the primer set for *FOSB* was optimised, the expression levels in MDBK cells were low. Further optimisation was performed by altering the concentration of cDNA used in the reaction however this failed to improve the levels of *FOSB* observed in the RT-qPCR and as such the regulation of *FOSB* by Ov2 could not be investigated. In the study done by Parameswaran

et al. CASP8 was up-regulated two-fold in an A2 deletant virus compared to the wild-type or revertant viruses by RNA-Seq. However in an RT-qPCR performed in rabbit LGL cells no significant difference was observed (Parameswaran *et al.*, 2014). In MDBK cells nucleofected with an Ov2-EGFPN1 construct there was no difference in the expression of *CASP8* compared to MDBK cells nucleofected with a pEGFPN1 construct. In the study by Parameswaran *et al. JAG1* was down-regulated twenty-seven-fold in an A2 deletant virus compared to the wild-type or revertant viruses by RNA-Seq. RT-qPCR for *JAG1* was not performed in the described study. In MDBK cells nucleofected with an Ov2-EGFPN1 construct the expression of *JAG1* was down-regulated 25% compared to MDBK cells nucleofected with an EGFPN1 construct. The Parameswaran *et al.* study implies that A2 would up-regulate *JAG1* expression however in the study performed as part of this thesis Ov2 down-regulates *JAG1* expression. Obviously the two systems used are completely different in a number ways, including the species and type of cell lines used. The work done on A2 was in the context of the whole virus; as this method is not available for the study of OvHV-2 a different approach had to be taken. Work is ongoing to determine the effect of A2 on *JAG1* using an A2-pEGFPN1 construct nucleofected into MDBK cells. This work, in combination with the work performed in section 4.3 showing that *DLL1* is a target of OvHV-2-encoded miRNAs, is good evidence supporting the hypothesis that Notch signalling plays an important role during OvHV-2 biology. This will be discussed in further detail in chapter 6.

Lastly, immunoprecipitation and LC MS/MS was performed to identify potential interacting partners of Ov2. A number of compromises were made in the proteomic study of Ov2, including the number of replicates, quantitative nature of the mass spectrometry and cell line in which the immunoprecipitation was performed. The immunoprecipitation of Ov2 was only performed once, and therefore would need to be repeated multiple times before any real conclusions could be drawn. There were a large number of proteins identified that were present in both samples. Although the protein score does approximately relate to the abundance of the protein other things such as number of peptides matching the protein are taken into consideration. In this analysis, there are a number of proteins that have a differential protein score of more than two-fold in the Ov2 sample compared to the control. This indicates that if this was to be repeated, a quantitative approach might be useful in determining whether the proteins that have a higher score in the Ov2 sample are potential interacting partners. There were also a large number of ribosomal proteins identified in both the Ov2 and control samples; this is most likely due to not using microcentrifuge tubes that have low protein-binding properties during the immunoprecipitation. Also worth noting was the fact that Ov2 had a low protein score itself of 54. When the Ov2 protein sequence was probed for trypsin cleavage sites (after arginine or lysine residues) only four peptides of an optimal length for LC MS/MS (approximately 1 kDa) were observed. Two of the four peptides were detected in this analysis, indicating that perhaps Ov2 is not an ideal candidate for LC MS/MS. Another potential issue with this analysis is that the immunoprecipitation was performed in 293T cells as they transfect easily. 293T cells are not a physiologically relevant cell line and perhaps a better choice would have

been a lymphocyte cell line, particularly sheep and/or cattle lymphocytes. However, lymphocytes are difficult to transfect and therefore it may be difficult to get enough protein sample to perform the immunoprecipitation. Moreover, performing the immunoprecipitation in sheep or cattle cell lines has the added complexity of having less well annotated genomes which may limit the results.

Of the unique proteins identified in the Ov2HA-pcDNA3.1+ sample there are two proteins of particular interest; nucleoside diphosphate kinase B (NME2) and putative RNA-binding protein Luc7-like 1 (LUC7L). *NME2* belongs to a family of nucleoside diphosphate kinases which catalyse the transfer of γ-phosphates from nucleoside triphosphates to nucleoside diphosphates. NME genes are also involved in a number of cellular processes such as proliferation, differentiation, apoptosis and metastatic dissemination (Boissan *et al.*, 2009; Liu *et al.*, 2015b). A potential mechanism through which NME2 suppresses metastatic activity is through interaction and sequestering of Integrin cytoplasmic domain-associated protein 1 α, a negative regulator of cell adhesion (Fournier *et al.*, 2002). NME2 is also capable of binding BCL2-Like 10 (BCL2L10), which can act as either a pro- or anti-apoptotic protein depending on the cellular context, thus providing a mechanism for NME2 to regulate apoptosis (Kang *et al.*, 2007). Of particular interest to Ov2 biology is the observation that NME2 can act as a DNA-binding factor and play a role in transcription (Postel, 2003). Furthermore, it was shown that NME2 can bind to a specific G-quadruplex motif located in the c-myc promoter and induce c-myc expression (Berberich & Postel, 1995; Postel, 2003; Yao *et al.*, 2014). c-myc is a known oncogene that contributes to the development of many human cancers. It has been shown that deregulated Notch signalling pathways can induce c-myc expression in a T cell leukaemia mouse model (Sharma *et al.*, 2006). Other viruses including EBV and human papillomavirus (HPV) can interact with another NME protein NME1. EBNA3C and EBNA1 of EBV can interact with and suppress the activity of NME1 promoting metastasis and the E7 protein of HPV can also bind NME1 and regulate tumour cell motility (Kaul *et al.*, 2007; Mileo *et al.*, 2006; Subramanian *et al.*, 2001; Subramanian & Robertson, 2002). It could be possible that OvHV-2 uses Ov2 to regulate cell migration like EBV and HPV. Another possibility is that Ov2 could bind to NME2 and induce c-myc expression contributing to the lymphocyte proliferation observed in MCF. It is possible that this happens in cattle and not in sheep due to a dysregulation of OvHV-2-encoded miRNA expression, which leads to improper Ov2 expression in cattle. Obviously, to confirm interaction of Ov2 with NME2, further validation such as a co-immunoprecipitation or confocal microscopy to confirm co-localisation would be required.

The other unique protein identified, LUC7L belongs to a family of Luc7 protein homologues. Luc7 is an essential subunit of the yeast U1 small nuclear ribonucleic protein (snRNP) that plays a role in 5' splice site recognition. Luc7 mutants are unable to support any of the defined steps of splicing unless recombinant Luc7 protein is added (Fortes *et al.*, 1999). It is worth noting that LUC7L2 was identified in both control and Ov2 immunoprecipitated samples however the protein score was 2.66 fold higher

in the Ov2 sample compared to the control sample. LUC7L3 was also identified in the Ov2 immunoprecipitated sample however due to only have one unique peptide in the analysis it was excluded from the unique protein list. A list of proteins with only one unique sample in both the control and Ov2 samples can be found in Appendix 8. Moreover, *LUC7L2* was identified as a differentially enriched gene in the CLASH work performed previously (Riaz, 2014). Differentially enriched genes are those that were precipitated more in one sample compared to the other; in this instance *LUC7L2* was enriched sixty-four fold in the control sample compared to the sample expressing OvHV-2-miR-17-1, 17-2 and 17-3. If the OvHV2-miRNAs were targeting *LUC7L2* then it should be differentially enriched in the test sample compared to the control, however if there was no regulation of *LUC7L2* by the OvHV2-miRNAs similar levels of the transcript between the two samples should have been precipitated. This indicates that perhaps the OvHV-2-miRNAs are down-regulating an upstream regulator of *LUC7L2* expression. Little work has been done on the function of LUC7L and LUC7L3 in humans however it has been shown that LUC7L3 interacts with the splicing factors SR-related protein of 53 kDa (SRrp53) and Serine/Arginine-Rich Splicing Factor 1 (SRSF1), and contains two zinc finger motifs (Cazalla *et al.*, 2005). *LUC7L2* has been shown to be a frequently mutated gene in some myeloid malignancies (Singh *et al.*, 2013). It is thought to be involved in recognition of non-consensus splice sites and co-localises with the spliceosome marker U1-70K and is thus likely to associate with U1 (Howell *et al.*, 2007). However, in a number of studies that investigated the components of the spliceosome LUC7L2 was not present (Jurica & Moore, 2003). It is possible that LUC7L2 interacts with the spliceosome transiently and plays a potential auxiliary role in splicing through stabilisation of the spliceosome (Howell *et al.*, 2007). LUC7L2 was identified as a potential interacting partner of the HIV encoded protein Tat by LC MS/MS however this interaction was not further validated (Gautier *et al.*, 2009). The role that the potential interaction of Ov2 with the LUC7L proteins in OvHV-2 biology is unclear, as the majority of OvHV-2 ORFs are not spliced. Normally, splicing is required for efficient nuclear export through the recruitment of Aly, a protein that is associated with spliced mRNPs and recruits nuclear export factors (Zhou *et al.*, 2000). It is known that other γ-herpesviruses such as KSHV, bypass splicing by recruitment of ORF57, which binds to the 5' of viral mRNPs and directly recruits Aly facilitating nuclear export (Boyne *et al.*, 2008). Moreover, ICP27 of HSV-1 is known to inhibit cellular splicing through interactions with essential splicing factors named SR proteins; this interaction can affect the phosphorylation status of SR proteins and consequently impair the ability of these proteins to function in spliceosome assembly (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994; Sciabica *et al.*, 2003). ICP27 can also interact with spliceosome associated protein 145 (SAP145), which can also lead to an inhibition of splicing, as a mutated ICP27 which does not inhibit splicing showed a reduced interaction with SAP145 (Bryant *et al.*, 2001). Additionally, it has been shown that the HIV-encoded protein Vpr is also capable of inhibiting splicing through interaction with SAP145 (Hashizume *et al.*, 2007). It is possible that Ov2 functions to down-regulate splicing as a mechanism of host shut-off however as the study of OvHV-2 gene expression is limited by the lack of a propagation

system it could be difficult to demonstrate the function of an interaction between Ov2 and LUC7L proteins during infection.

The lack of an obvious transcription factor in the Ov2 mass spectrometry samples could be interpreted as an indication that Ov2 does not play a role in the manipulation of cellular gene expression, however this cannot be ruled out by the preliminary analysis done in this thesis. It is possible that Ov2 is only capable of forming homodimers and as such this analysis would not indicate whether cellular gene expression was regulated. Alternatively, chromatin immunoprecipitation assays with sequencing could be used to determing DNA binding sites for Ov2 in order to study Ov2 control of gene expression. Due to time restrictions, confirmation that Ov2 interacts with itself was not able to be performed, but work is ongoing to investigate this. It is also possible that Ov2 may require other OvHV-2-encoded proteins in order to induce target gene expression; another viral protein Ov6 also contains a bZIP domain and so an interaction between Ov2 and Ov6 is possible. Alternative methods for identifying Ov2 binding partners could include yeast two-hybrid screens, or chemical cross-linking of proteins followed by mass spectrometry. Another potential hypothesis is that Ov2 only regulates virus gene expression; work is ongoing in the lab conducting promoter assays with Ov2 and a number of virus promoters. This preliminary analysis into the function of Ov2 has yielded some interesting results and further investigation is required to fully elucidate the function of Ov2.

Chapter 6: Concluding Remarks

This project had two major aims; firstly to validate the expression of previously predicted OvHV-2-encoded miRNAs and secondly to identify and characterise cellular and viral targets of OvHV-2-encoded miRNAs. The aims of this project were successful in that the expression of a total of 35 OvHV-2-encoded miRNAs was demonstrated and that both cellular and virus targets of these virus-encoded miRNAs were identified and validated.

The first aim of this project was described and discussed in Chapter 3. Any further characterisation of the validated OvHV-2-encoded miRNAs, such as how and when they are expressed during infection, was hindered by the lack of an appropriate model. Unlike, AlHV-1, OvHV-2 does not have a system for virus propagation *in vitro*. Moreover, there is a lack of a suitable model to study the natural infection of OvHV-2; a population of uninfected sheep can be raised in the correct conditions, however this is very expensive and would be out with the scope of this project (Meier-Trummer *et al.*, 2010). The rabbit model of MCF could potentially be used to investigate the temporal expression of OvHV-2-encoded miRNAs however this was also out with the scope of this project. Based on the fact that miRNAs encoded by other gammeherpesviruses are expressed during latency, and that the majority of OvHV-2-encoded miRNAs are clustered as in for example KSHV, it could be predicted that expression of OvHV-2-encoded miRNAs would also be during latency. Therefore, it may be more biologically relevant to examine the temporal expression of OvHV-2-encoded miRNAs in sheep, which as mentioned above, is difficult. However, given the evidence presented in section 1.5.4 where subclinical infection of cattle can occur, it is also likely that there is latent infection of OvHV-2 in cattle and as such cattle could also be used to examine temporal expression of virus-encoded miRNAs during OvHV-2 infection.

BJ1035 cells were the most readily available source of OvHV-2-infected material during this project and so were used to validate the expression of OvHV-2-encoded miRNAs. Additionally, as the initial RNA-seq experiment was performed in BJ1035s it was logical to perform the validation in the same cell type. However, it is possible that the OvHV-2 genome codes for a number of miRNAs not identified in the BJ1035 RNA-Seq experiment. Furthermore, as OvHV-2 will have co-evolved with sheep, there may be miRNAs that are only expressed in the natural host. Therefore, a criticism of this project could be that no sheep tissue was examined for the expression of OvHV-2-encoded miRNAs. Also, as BJ1305 cells have been in culture for many years, it is likely that they do not represent OvHV-2 infection in cattle. Therefore, tissue from an OvHV-2-infected cow could also have been examined for the expression of OvHV-2-encoded miRNAs.

The second aim of this project was split into two strands; identification and characterisation of cellular targets was described in Chapter 4 and the identification and characterisation of the OvHV-2-encoded protein Ov2 as a target of OvHV-2 encoded miRNAs was described in Chapter 5. The individual details of these results are discussed at the end of the relevant chapters. Studies on other herpesviruses have taken advantage of being able to manipulate the virus and construct viruses that lack one or more

miRNAs in order to identify potential targets. As this is not an option with OvHV-2-encoded miRNAs a different approach was taken. Dr Riaz performed CLASH (described in section 4.1) and had also performed a microarray to identify potential targets of ovhv2-miR-17-1, ovhv2-miR-17-2 and ovhv2-miR-17-3 so these methods were not used in this thesis (Riaz, 2014). *DLL1*, a target of ovhv2-miR-17-2 identified in the CLASH study performed by Dr Riaz, was confirmed by luciferase assay (see section 4.3.6). Based on homology between the cellular miRNA miR-216a and ovhv2-miR-73-1 *PTEN* and *YB-1* were investigated as targets. Due to difficulties in the luciferase assay, which were described in section 4.6.2, a GFP-reporter system was used to demonstrate that ovhv2-miR-73-1 does not target *PTEN* or *YB-1* (section 4.4.5). Lastly, a bioinformatic approach using RNAHybrid was used to identify MHC class II genes and the OvHV-2-encoded protein Ov2 as potential targets of OvHV-2-encoded miRNAs. Two genes, (ovine *DRA* and ovine *DQB*) were confirmed as targets of ovhv2-miR-17-25 and ovhv2-miR-17-9 respectively by luciferase assays. Further characterisation of these targets was not performed, due to difficulties in finding appropriate cell lines that could be used for study and also technical difficulties in the luciferase reporter system used for validation.

There are a number of difficulties in working with a virus that infects sheep and cattle, which are not limited to, but include the lack of available reagents such as antibodies, physiologically-relevant cell lines and incomplete/inaccurate annotation of the sheep and cattle genome assemblies. Although the number of reagents available is increasing, there are still far fewer reagents available compared to the number of reagents available for work in human cells or mice. Attempts to use biologically relevant cell lines were made during this thesis; initially bovine turbinate cells were used instead of MDBK cells for the work described in section 5.4.2, however suitable levels of transfection efficiency could not be achieved, and optimisation of any nucleofection protocol was not viable in the time available. Although the MDBK cells do not transfect efficiently, there is an existing nucleofection protocol provided by Lonza (see section 2.1.9) to yield the highest nucleofection efficiency and minimal cell death. Furthermore work in lymphocytes, which are obviously of high biological relevance, is often difficult. Again there are problems in achieving high transfection efficiency, and it can be often difficult to grow these cells in culture in the amounts required for experimental work. Lastly, incorrect annotation of the sheep genome was a major problem encountered in the validation and characterisation of *DLL1* as a target of ovhv2-mIR-17-2. Additionally, the immunoprecipitation and mass spectrometry analysis of Ov2 described in section 5.4.3 was performed in a human cell line to increase the chance of detecting proteins of interest due to the lack of a well annotated genomes for sheep and cattle.

In addition to the technical problems and limitations described above, the methods of identification and validation of targets of OvHV-2-encoded miRNAs used in this thesis have their limitations. RNAHybrid was chosen as the primary bioinformatic programme used in OvHV-2-encoded miRNA target identification as it was deemed to be the only available option. Although it would have been advantageous in eliminating false-positives, the use of multiple prediction methods utilising different

strategies of prediction was not possible for the purposes of this thesis (Witkos *et al.*, 2011). This was mainly because many other programmes can only predict targets of miRNAs within a single species (e.g. human miRNAs targeting human mRNAs). Depending on the programme used this can be extended to a few other species such as mouse and frog. As part of this project was identifying cellular targets of virus-encoded miRNAs many other prediction programmes (listed in section 1.6.4) were not suitable. Furthermore, many of these programmes base target prediction on evolutionary conservation of a target site, which again is not a suitable approach when investigating virus-encoded miRNAs that show little evolutionary conservation. There are currently two prediction programmes that are capable of predicting host targets of a virus-encoded miRNA. These are RepTar (Elefant *et al.*, 2011) and vHOT DB (http://best.snu.ac.kr/vhot/). RepTar can only predict human targets of HCMV, KSHV and EBV encoded miRNAs and mouse targets of MCMV and MHV-68; it is therefore not suitable for the purposes of this thesis. vHOT DB can predict targets from any virus-encoded miRNA listed in miRbase in the following species: human, mouse, cow, rhesus monkey, rat and the viruses themselves. As the OvHV-2-encoded miRNAs are currently not listed in miRbase and prediction of targeting in sheep is not available using vHOT DB it is also not suitable for the purposes of this thesis.

RNAHybrid predicts miRNA targets based on thermodynamic interactions. The limitation in using RNAHybrid is that genome wide analysis cannot be attempted as the potential target mRNA must be specified in the analysis. A number of potential targets may also be missed by the restrictions imposed on the search such as perfect complementarity in the seed sequence and whether or not G:U pairing is allowed in the seed sequence. If such restrictions are not imposed then the list of potential target sites is increased, which consequently decreases the chance of identifying a functional target site. Additionally, using only thermodynamic characteristics to predict target sites can lead to a high rate of false positives. However, for the purposes of this project RNAHybrid was the most suitable programme for target identification and did identify a number of targets that were successfully validated using a biochemical method. One of the methods used for validation of predicted targets in this project was the luciferase assay. This assay provides a direct link between the target site and the expression of the reporter gene used, in this case *Renilla* luciferase. However, off target effects of the OvHV-2-encoded miRNAs were found to be fairly common meaning that the luciferase assay was not a suitable approach to use in some instances. In the majority of cases in this project, to avoid losing the context of the target site, the entire 3'UTR of the gene of interest was cloned into the psiCHECK-2 vector. This was not always possible, for example in the case of *PTEN* the 3'UTR was too long. Additionally, the target site confirmed by luciferase assay in *DLL1* was cloned into the 3'UTR of *Renilla* luciferase, however this target site is present in the coding region of *DLL1*. It could be possible to engineer the target site into the coding region of *Renilla* luciferase however this was not attempted in this project.

Validation of Ov2 as a target of OvHV-2-encoded miRNAs was performed using a GFP reporter system and western blotting, with site-directed mutagenesis confirming the target site for ovhv2-miR-61-1. The

target site for ovhv2-miR-17-10 could not be confirmed using site-directed mutagenesis. Characterisation of the down-regulation of Ov2 at the transcript level could not be performed due to the fact that Ov2 mRNA was unable to be detected by PCR even when overexpressed. The levels of protein in similar overexpression experiments were confirmed by western blotting so the constructs used in this project were functional. A number of different primer sets were used and all were capable of amplifying Ov2 from genomic DNA possibly indicating that the Ov2 transcript has a very short half-life. In fact, many regulatory proteins including transcription factors often have a short half-life (Lamph *et al.*, 1988; Rogers *et al.*, 1986; Treier *et al.*, 1994).

The final part of this thesis involved the functional characterisation of Ov2 (section 5.4). Immunofluorescence confirmed the nuclear localisation of Ov2, RT-qPCR analysis of MDBK cells nucleofected with an Ov2-EGFPN1 construct demonstrated the down-regulation of expression of *JAG1*. Immunoprecipitation and subsequent mass spectrometry analysis revealed the potential interaction of Ov2 with NME2 and members of the LUC7L family of proteins. Validation of this work is obviously required to confirm any interaction. However this potential interaction, along with the identification of LUC7L2 as under-represented in SEFs transduced with a lentivirus containing ovhv2-miR-17-1, ovhv2-miR-17-2 and ovhv2-miR-17-3 compared to SEFs transduced with the control lentivirus plasmid in the CLASH study performed by Dr Riaz are promising pieces of evidence towards the hypothesis that OvHV-2 disrupts cellular splicing that warrant further investigation.

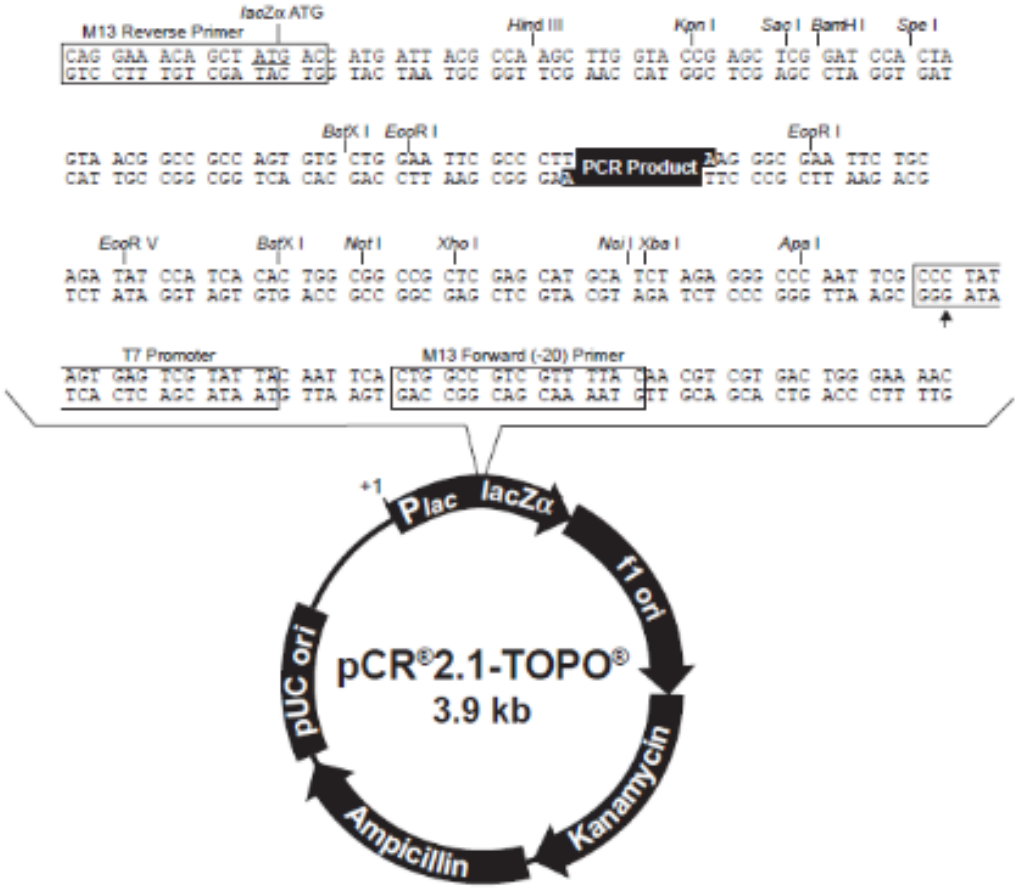
Notch signalling emerged as an area of potential regulation by OvHV-2 from the results of this thesis. Two ligands for Notch signalling, delta1 and jagged1 were identified to potentially be regulated by OvHV-2. It could be hypothesised that Notch signalling would be down-regulated in sheep in order to promote latency. As the virus will have co-evolved with sheep it is likely that cellular pathways in sheep are the "original" targets of OvHV-2-encoded proteins and miRNAs. Some of these pathways may be regulated in the same way in MCF-susceptible hosts however it is possible that due to slight differences in sequence between the sheep and cattle genome differential targeting occurs and, as a result of this dysregulation, MCF develops. It is also possible that there is a differential regulation of OvHV-2 gene expression, including expression of miRNAs, which lead to altered targeting of pathways and development of MCF. Due to the importance of Notch signalling during development in determination of cell fate (reviewed in Andersson *et al*. 2011) and the fact that constitutive activation of Notch in T cells causes acute lymphoblastoma, Notch signalling is under tight regulation (Schweisguth, 2004). OvHV-2-encoded miRNAs may contribute to this regulation during infection, as it is possible that Notch signalling is required at certain points during the lifecycle of OvHV-2. ovhv2-miR-17-2 appears to have a direct effect on the levels of *DLL1* as confirmed by luciferase assay, whereas ovhv2-miR-17-10 and ovhv2-miR-61-1 may be able to indirectly regulate the expression of Jagged through regulation of Ov2 expression. As it is not known when these miRNAs are expressed during infection, the context of regulation of Notch signalling is unclear. As there is no cell culture model of OvHV-2, and

investigating the role OvHV-2 plays in Notch signalling during infection of previously uninfected sheep would be difficult and very costly, any downstream effects OvHV-2-encoded miRNAs would have as a consequence of targeting Notch receptor ligands would be difficult to study. However, the rabbit model of MCF could be used as an alternative to look at the influence of OvHV-2 on Notch signalling in MCF-susceptible species.

Results obtained from this thesis present novel mechanisms of potential regulation of cellular processes by OvHV-2-encoded miRNAs. These may contribute to the differential outcomes observed in OvHV-2 infection in sheep and MCF-susceptible species. Although an incomplete story, mainly due to restrictions in both time and the availability of appropriate systems for the study of OvHV-2, this thesis paves the way for future investigations into the role of OvHV-2-encoded miRNAs in OvHV-2 biology.

Appendix 1: Vectors and Plasmids

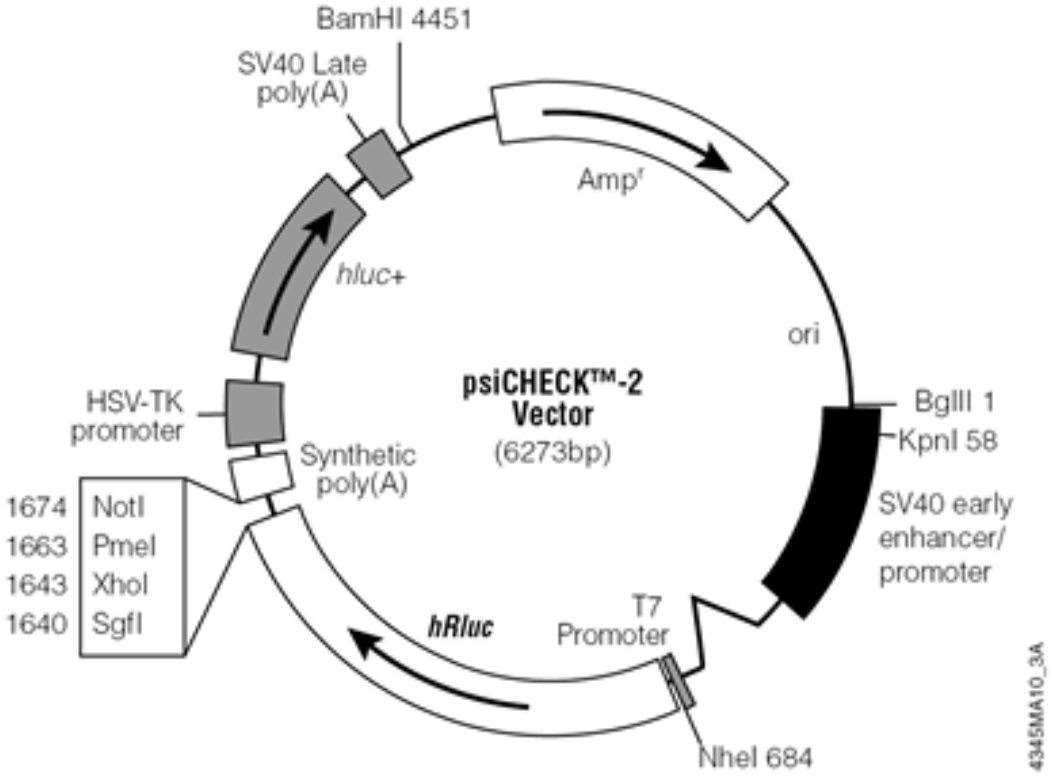
pCR 2.1-TOPO



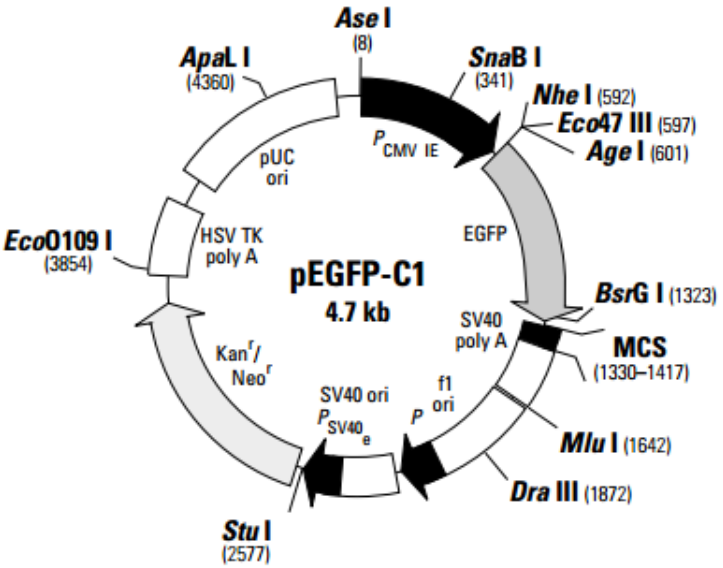
Comments for pCR®2.1-TOPO®
3931 nucleotides

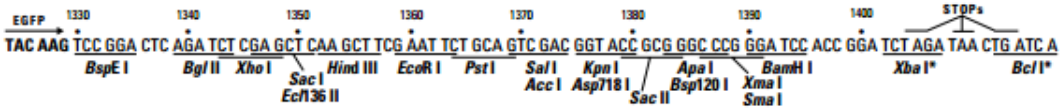
LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

psiCHECK™-2 Vector



pEGFP-C1


Ase I
(8)
ApaL I
(4360)
SnaB I
(341)
Nhe I (592)
Eco47 III (597)
Age I (601)
P CMV IE
pUC
ori
EGFP
Eco0109 I
(3854)
HSV TK
poly A
pEGFP-C1
4.7 kb
BsrG I (1323)
SV40
poly A
MCS
(1330–1417)
Kanr/
Neor
SV40 ori
P SV40 e
f1
ori
P
Mlu I (1642)
Dra III (1872)
Stu I
(2577)


EGFP
1330
1340
1350
1360
1370
1380
1390
1400
STOPs
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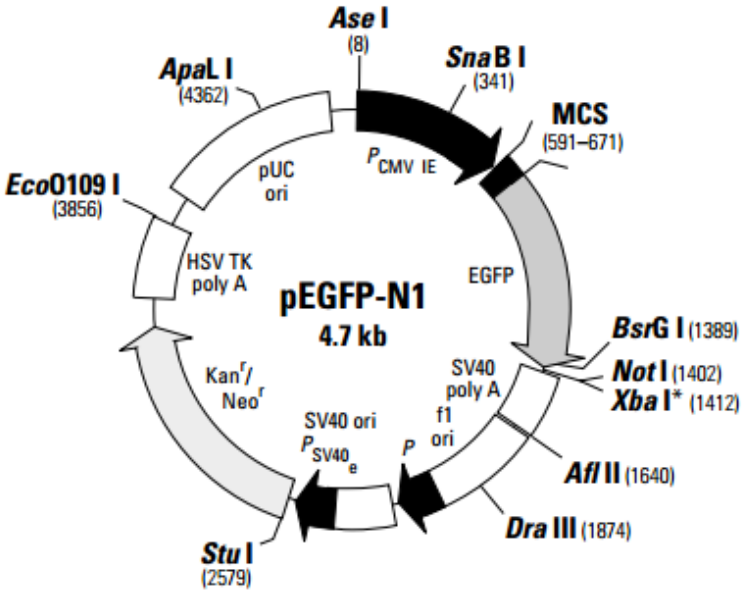
BspE I
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
BamH I
Xma I
Sma I
Xba I*
Bcl I*

pEGFP-N1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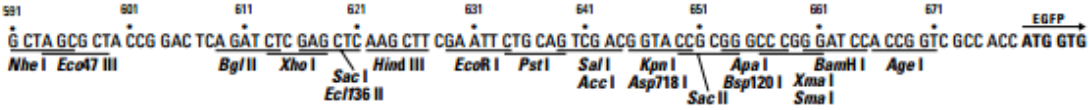
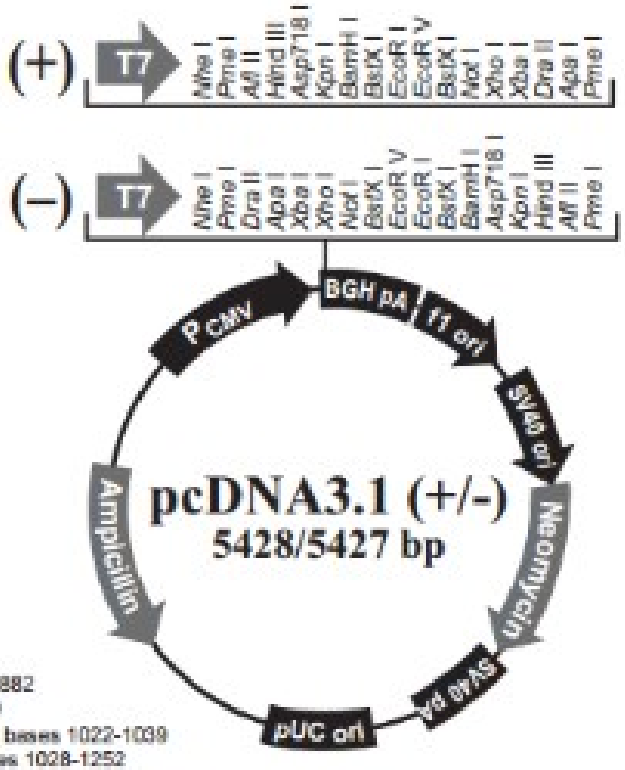
SnaB I
(341)
MCS
(591–671)
ApaL I
(4362)
Eco0109 I
(3856)
pUC
ori
P CMV IE
HSV TK
poly A
EGFP
pEGFP-N1
4.7 kb
BsrG I (1389)
Not I (1402)
Xba I* (1412)
Kanr/
Neor
SV40
poly A
f1
ori
SV40 ori
P SV40e
P
AflII (1640)
Dra III (1874)
Stu I
(2579)


591
601
611
621
631
641
651
661
671
EGFP
G CTA GCG CTA CCG GAC TCA GAT CTC GAG CTC AAG CTT CGA ATT CTG CAG TCG ACG GTA CCG CGG GCC CGG GAT CCA CCG GTC GCC ACC ATG GTG
Nhe I Eco47 III
Bgl II
Xho I
Sac I
Ecl136 II
Hind III
EcoR I
Pst I
Sal I
Acc I
Kpn I
Asp718 I
Sac II
Apa I
Bsp120 I
BamH I
Xma I
Sma I
Age I

pcDNA3.1 +



Comments for pcDNA3.1 (+)
5428 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation sequence: bases 1028-1252
f1 origin: bases 1298-1726
SV40 early promoter and origin: bases 1731-2074
Neomycin resistance gene (ORF): bases 2136-2930
SV40 early polyadenylation signal: bases 3104-3234
pUC origin: bases 3617-4287 (complementary strand)
Ampicillin resistance gene (*bla*): bases 4432-5428 (complementary strand)
ORF: bases 4432-5292 (complementary strand)
Ribosome binding site: bases 5300-5304 (complementary strand)
bla promoter (P3): bases 5327-5333 (complementary strand)

Appendix 2: Primers and Oligonucleotides

miRNA RT-qPCR primers

ovhv2-miR	Primer sequence	RT-PCR Annealing temperature (°C)
Ov2-1	ATGCTTGTTTAGGCCC	48
17-30	TTTGGGTGTCTCCTG	46
17-28	TCTAGGTTGCATTTTG	44
17-27	CCCACATTTAAGGTG	44
17-26	ATATTCGTTTAGACG	40
17-25	CAATGCTGCTTTGGTG	48
17-24	GGGTTCCTCGAGTGG	52
17-23	ATACACACTGAAAGAGC	48
17-22	ATAAGGCCAACACTAG	46
17-21	AAGCACCTTGGGTGATGTC	58
17-19	AAGCATAGCTGGGAGTG	52
17-18	TAGTAGTCCGTTAACG	46
17-16	TAAACTGGTGGTAGG	44
17-15	TAGCAGTTATGCAGGTATC	54
17-14	TGGCATTTCCAGGAGCCTG	60
17-13	TTGGGTCCAACATGAGA	50
17-12	TATGTCAGAAGTGAAGC	48
17-11	TGGTTTGCATCTGCAC	52
17-9	TAGAGTTACTAAGGATTC	48
17-8	AATCGCCGGTGGCCTTC	56
17-7	TATAGACGGGTATGC	44
17-5	CCTTTTTGGTGAGTTGC	50
17-4	GATTTGATAAAGCCTGC	48
17-2	ACCCCGGGGGTATGTG	54
24-1	GAGCAGTACTACACAGCAG	58
61-1	TTGGGGACGTGCTGGCTG	60
Group 1	TCCCGAGATGTCGGG	50
Group 3	TTGCGGGGAAGGCCGC	56
Group 13	GATGTAGGACAGGCCGC	56
Group 34	TTTGGGTGTCTCCTG	46

Group 95	GCTGCGCTCGCTTGGG	56
Group 128	ACGGGTGAGGTGGGGC	56
Group 181	CATTGGGGAGGCCGG	52
Group 182	AGTGGATGTAGTCCTGG	52
Group 3p	TCCCGAGATGTCGGG	50
Group 64p	GGGCGGCTTAGTAAC	48
Group 96p	TTGATAGCAGGATGTGC	50

miRNA specific cDNA primers

ovhv2-miR	PCR Forward Primer Sequence	Reverse Transcription primer sequence
17-19	GCGGCGAAGCATAGCTGGGAGTG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACTCTAGA
17-12	GCGGCGTATGTCAGAAGTGAAG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACTCTCAG
17-7	GCGGCGGTATAGACGGGTATG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCGGCAG
61-1	GCGGCTTGGGGACGTGCTGGCTGA	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACACGTCG
73-1	GCGGCGTAATCTCTGCTCCAATT	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACATTTAC
Group 1	GCGGCGGTCCCGAGATGTCGG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCCAGTC
Group 3	GCGGCGTTGCGGGGAAGGCCGC	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACAAACAC
Group 13	GCGGCGGATGTAGGACAGGCCG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCATCAG
Group 34	GCGGCGTTTGGGTGTCTCCTG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACAGATGA
Group 95	GCGGCGCTGCGCTCGCTTGGGGCC	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACTTGAGG
Group 128	GCGGCGGACGGGTGAGGTGGG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCCCTGC
Group 181	GCGGCGCATTGGGGAGGCCGG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCTTCCC
Group 182	GCGGCAGTGGATGTAGTCCTGGAAG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACTAAAGC
Group 3p	GCGGCGGTCCCGAGATGTCGG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCCAGTC
Group 64p	GCGGCGGGGCGGCTTAGTAA	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCACATG
Group 96p	GCGGCGTTGATAGCAGGATGTG	GTCGTATCCAGTGCAGGGTCCCGAGGTATTCGCACTGGATACGACCAGGCA
	PCR Universal Reverse Primer	
	GTGCAGGGTCCGAGGT	

Target Gene Primers

Gene	Primer Sequence
DLL1 1970-2119 "a" Forward	ACTACTGCACGCACCACAAG
DLL1 1970-2119 "a" Reverse	GGTCCTGAGTGACGGAGAAG
DLL1 1538-1756 "b" Forward	CAGAAAGACTTATCAGCCGC
DLL1 1538-1756 "b" Reverse	TGCAGACTTTCTCCCCTCTC
DLL1 2415-2475 Xho1 "c" Forward	CTCGAGGACGACTGTGCCTCCTCC
DLL1 2415-2475 Not1 "c" Reverse	GCGGCCGCGTCTGCCTTCTTGTTGGTGT
DLL1 RACE 5'	TAATTCTCGCGCCGGCCCCCGGGTGT
DLL1 RACE 3' "d"	TTCCAAGGGAGGGCGCCCCGACA
DLL1 2711-2931"e" Forward	CCACGCAGATCAAGAACACC
DLL1 2711-2931 "e" Reverse	CTGCTCCTCGGATATGACGT
GAPDH Sheep/Cattle RT-qPCR Forward	GGTGATGCTGGTGCTGAGTA
GAPDH Sheep/Cattle RT-qPCR Reverse	TCATAAGTCCCTCCACGATG
Jagged Cattle/Sheep RT-qPCR Forward	AAGTGCAAGAGTCAGTCGGG
Jagged Cattle RT-qPCR Reverse	AATGCACGCGTAGGAGTTCA
Jagged Sheep RT-qPCR Reverse	GATGCACGCGTAGGAGTTGA
Caspase 8 sheep/cattle RT-qPCR Forward	TCACAATCGCTTCCAGAGGA
Caspase 8 sheep/cattle RT-qPCR Reverse	TATCCCCGAGGTTTGCTTGT

Ovine MHC DRA Xho1 Forward	AACTCGAGCACCTTTTCCGCAAGTTCCA
Ovine MHC DRA Not1 Reverse	AAGCGGCCGCAGACCCACTTGAAGTTTAC
Ybx1 3'UTR Xho1 Forward	AACTCGAGACCATCTCTACCATCATCCGG
Ybx1 3'UTR Not1 Reverse	AAGCGGCCGCACAGGTGCTTGCAGTTTGTT
Ybx1 pEGFP-C1 Xho1 Forward	AACTCGAGCTCAATACACCTTTA
Ybx1 pEGFP-C1 Kpn1 Reverse	AAGGTACCGCGGCCGCGTAAACT
PTEN pEGFP-C1 Xho1 Forward	AACTCGAGATATACAATATTTTG
PTEN pEGFP-C1 Kpn1 Reverse	AAGGTACCGCGGCCGCGGAATAA
Ov2 ovhv2-miR-17-10 binding site mutagenesis Forward	GCTCCTGTAACCTCTTCTTAAAATTGCGTGATGCCCTTCTATTTGTGCTCCTGC
Ov2 ovhv2-miR-17-10 binding site mutagenesis Reverse	GCAGGAGCACAAATAGAAGGGCATCACGCAATTTTAAGAAGAGGTTACAGGAGC
Ov2 ovhv2-miR-61-1 binding site mutagenesis Forward	CATCCACTCCCACCTACCTGAGGTGACCATAAATGTTGTTCGGAGTTGTAAAAATCATTTGT
Ov2 ovhv2-miR-61-1 binding site mutagenesis Reverse	ACAAATGATTTTTACAACTCCGAACAACATTTATGGTCACCTCAGGTAGGTGGGAGTGGATG

Target Gene Oligonucleotides

Gene	Oligonucleotide Sequence
DLL1 Forward	TCGAGGGCCTTCCGCTACCTGTGCGAGTGTGCCCGGGGCTACGGGGGACAGGCTGGGAAGGGG GGCCTTCCCGGGGAGGGCGCCCCGACACCCGGGGGCCGGCGCGAGGC
DLL1 Reverse Complement	GGCCGCCTCGCGCCGGCCCCCGGGTGTCGGGGCGCCCTCCCCGGGAAGGCCCCCCTTCCCAGC CTGTCCCCCGTAGCCCCGGGCACACTCGCACAGGTAGCGGAAGGCCC
DLL1 1st site mutated Forward	TCGAGGGCCTTCCGCTACCTGTGCGAGTGTGGGATCCCCTACGGGGGACAGGCTGGGAAGGGG GGCCTTCCCGGGGAGGGCGCCCCGACACCCGGGGGCCGGCGCGAGGC
DLL1 1st site mutated Reverse Complement	GGCCGCCTCGCGCCGGCCCCCGGGTGTCGGGGCGCCCTCCCCGGGAAGGCCCCCCTTCCCAGC CTGTCCCCCGTAGGGGATCCCACACTCGCACAGGTAGCGGAAGGCCC
DLL1 3rd site mutated Forward	TCGAGGGCCTTCCGCTACCTGTGCGAGTGTGCCCGGGGCTACGGGGGACAGGCTGGGAAGGGG GGCCTTCCCGGGGAGGGCGCCCCGACAGGATCCCGCCGGCGCGAGGC
DLL1 3rd site mutated Reverse Complement	GGCCGCCTCGCGCCGGCGGGATCCTGTCGGGGCGCCCTCCCCGGGAAGGCCCCCCTTCCCAGC CTGTCCCCCGTAGCCCCGGGCACACTCGCACAGGTAGCGGAAGGCCC
DLL1 1st and 3rd sites mutated Forward	TCGAGGGCCTTCCGCTACCTGTGCGAGTGTGGGATATCCTACGGGGGACAGGCTGGGAAGGGG GGCCTTCCCGGGGAGGGCGCCCCGACAGGATCCCGCCGGCGCGAGGC
DLL1 1st and 3rd sites mutated Reverse Complement	GGCCGCCTCGCGCCGGCGGGATCCTGTCGGGGCGCCCTCCCCGGGAAGGCCCCCCTTCCCAGC CTGTCCCCCGTAGGATATCCCACACTCGCACAGGTAGCGGAAGGCCC
MHC class II bovine DQA1 Forward	TCGAGTTGCATACCATGGAATTGCTACGGTATTCCACCCAATTACCTAATCTCTAGC

MHC class II bovine DQA1 Reverse Complement	GGCCGCTAGAGATTAGGTAATTGGGTGGAATACCGTAGCAATTCCATGGTATGCAAC
PTEN Forward	TCGAGATATACAATATTTTGTAATGCTGCACAGGAAATTTCAATTTGAGATTCTACAGTAAGCG GTTTTTTTTTCTTTAAAAATTTATGATCTTATTCGC
PTEN Reverse Complement	GGCCGCGAATAAGATCATAAATTTTTAAAGAAAAAAAAAACCGCTTACTGTAGAATCTCAAATT GAAATTTCCTGTGCAGCATTACAAAATATTGTATATC
Ybx1 100bp Forward	TCGAGCTCAATACACCTTTAAAGGTTTTTAAATTGTTTCATATCTGGTCAAGTTGAGATTTTTAA GAACCTCATTTTTAATTTGTAATAAAAGTTTACGC
Ybx1 100bp Reverse Complement	GGCCGCGTAAACTTTTATTACAAATTAAAAATGAGGTTCTTAAAAATCTCAACTTGACCAGATA TGAAACAATTTAAAAACCTTTAAAGGTGTATTGAGC

Appendix 3: *DLL1* Alignments and Sequences

Sheep *DLL1* alignments

```
Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          ATGGACCCACACTCCCAACCACTGCAGATCCTGCCGGCCCTGGGCTGGCCTGGGAAGCTG     60
Sheep_NCBI_New      ------------------------------------------------------------     0

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          TGCGTGTTTCTGATGCAGAAGACGGACTTCACAGAACCCCCTCAACCGCAGGCCAGGGTG     120
Sheep_NCBI_New      ------------------------------------------------------------     0

Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GCGCCGGGGGGAGCAGGCAGGGCGCACAGCTGTGGGGTCGAGCGGTCAGGGAGACGCGGT     180
Sheep_NCBI_New      ------------------------------------------------------------     0

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          TCAGGGACCGCGACGGCGCCGAATCGCGAGTGCACCCTCAGCGCCGAGGCGACAGCCCCG     240
Sheep_NCBI_New      ------------------------------------------------------------     0

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep NCBI          CAGGCCCAGCCCTACCTAGAGACGCGCCAGGACACCGGAGACGCGCCCACGCCGCCCTCG     300
Sheep NCBI New      ------------------------------------------------------------     0

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GCCCTCAGCAGCTGTGCGCACTTCCGCTTCCCGTCGCGAGACCGCCCCGCCCGCGCGGGG     360
Sheep_NCBI_New      --------------------ATGTGGCTCAGCAGCG-----------CGCCCCCGCCGGC     29

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GCGGGGCCTGCGCGGCCGGCCGGGAGGCGCCGGGAGCGCCACGCGCAGGCGCCTGAGAGC     420
Sheep_NCBI_New      TCCCACCCCCCA----CCGCCACCCCCAGCCCAGACCGACATC--------------AGA     71

Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GCGTGGCGGTGCCGGCTCCGGCTCTGCGCCCGTTGCCGCGGAAACCGCGCTGTGCTTCCG     480
Sheep_NCBI_New      CGTTGCAGGAACCCACGCCC--AGTTCAGCAGGAAACAGGTGCCCACATCTGTTCCTGGG     129

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          AGGTCGGGGTCACGACTCGCTCGGCTTCCGCAGGCGTACCCGTTGGTGCTGTGGGCGGCG     540
Sheep_NCBI_New      CGGGCAGATGCTCGCAGAAAGGAGGTGGGAGGCCGGGCAGGACGGCGCAGCCGCGCGGAG     189

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          CAGCGGCGGTGGGGCCCCGCCTCGGTGCAGGTGACCGCGTGCGCCCTGTGCCGCGCGCCC     600
Sheep NCBI New      CCGCTCGGGTGGGGCCCCGCCTCGGTGCAGGTGACCGCGTGCGCCCTGTGCCGCGCGCCC     249

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GGCGACCAGGGCTGGCCCGTCGACCTCGCCATCCCAGCCCGCGCGCATGTCCCTGCGACG     660
Sheep_NCBI_New      GGCGACCAGGGCTGGCCCGTCGACCTCGCCATCCCAGCCCGCGCGCATGTCCCTGCGACG     309

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GGTGCCCGCCGCCGCCACCCACTCCAAGCCACCCTCTGGAGCGCCCACCGGCGCCGCACC     720
Sheep_NCBI_New      GGTGCCCGCCGCCGCCACCCACTCCAAGCCACCCTCTGGAGCGCCCACCGGCGCCGCACC     369

Sheep Ensembl       ------------------------------------------------------------     0
Sheep NCBI          CCCTCCAGGCAGCCCTCCGGAGCGCCTGGCCCGGCGCGTTCAGTCGGGGGCCGCTCGGGC     780
Sheep_NCBI_New      CCCTCCAGGCAGCCCTCCGGAGCGCCTGGCCCGGCGCGTTCAGTCGGGGGCCGCTCGGGC     429

Sheep Ensembl       ------------------------------------------------------------     0
Sheep NCBI          GGGAGCGCGGAGCAGCTGCGCGGTCATCTGATCCGGACAAAGGCGCCCGGAGCCGCGCCA     840
Sheep_NCBI_New      GGGAGCGCGGAGCAGCTGCGCGGTCATCTGATCCGGACAAAGGCGCCCGGAGCCGCGCCA     489

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GTGAAAGTCACGTTTTGCCAGGACAGTGCAGTCCTGACAGAGAGGCCGGCTGCAGCACAC     900
Sheep NCBI New      GTGAAAGTCACGTTTTGCCAGGACAGTGCAGTCCTGACAGAGAGGCCGGCTGCAGCACAC     549

Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          CTACAGCGAGGCCTCAGTGACCGGGCGCCCTGTGCGGTACTGCTCATCAACGTGACCTTT     960
Sheep_NCBI_New      CTACAGCGAGGCCTCAGTGACCGGGCGCCCTGTGCGGTACTGCTCATCAACGTGACCTTT     609
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```
Sheep_Ensembl       ------------------------------------------------------------     0
Sheep NCBI          ACTCCACGCCCACACCTTTCACCCCGGATGACGGTGCCCCGGGTGTTGGGGGTGGGGGAG     1020
Sheep NCBI New      ACTCCACGCCCACACCTTTCACCCCGGATGACGGTGCCCCGGGTGTTGGGGGTGGGGGAG     669


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          ATGGAAGGAGGAGCAGATAGGGGTGAGATAGAGACCTCAACCTATGCGGGCGGCCGGGAC     1080
Sheep_NCBI_New      ATGGAAGGAGGAGCAGATAGGGGTGAGATAGAGACCTCAACCTATGCGGGCGGCCGGGAC     729


Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          CGCCGCCCCCGAGTGAGCCGGCCGGGCCGATCCTGCTCCACCGCCCCGGATTACCGCCTC     1140
Sheep_NCBI_New      CGCCGCCCCCGAGTGAGCCGGCCGGGCCGATCCTGCTCCACCGCCCCGGATTACCGCCTC     789


Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          GCGCGCGGACCTAAGGAACCTGGGCCTCCGGAAGAGCCGGAGCCGGACCCTTCAGGAGAG     1200
Sheep_NCBI_New      GCGCGCGGACCTAAGGAACCTGGGCCTCCGGAAGAGCCGGAGCCGGACCCTTCAGGAGAG     849


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep NCBI          AAGTCCGCATTATCTCGGCCCGAGGCAGAGCGGCGGCGCTTTCCTGCCCGAGCTCCCGGG     1260
Sheep NCBI New      AAGTCCGCATTATCTCGGCCCGAGGCAGAGCGGCGGCGCTTTCCTGCCCGAGCTCCCGGG     909


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep NCBI          GACACCTTCCTCCTCCAGCGTCCCCTGACGACTTCGGAAGACAAGGCGGGGGGGCCCTCC     1320
Sheep NCBI New      GACACCTTCCTCCTCCAGCGTCCCCTGACGACTTCGGAAGACAAGGCGGGGGGGCCCTCC     969


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          AAGCCCGGGTGGCTCACAGCCGGCGGCAGCGGCGAGGGGGTGTTGCGCGGGGTGACAGCC     1380
Sheep_NCBI_New      AAGCCCGGGTGGCTCACAGCCGGCGGCAGCGGCGAGGGGGTGTTGCGCGGGGTGACAGCC     1029


Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          CCGGTTGAGAGCGGCGGAGAGGCCAAGCACCCGGGACGCTTCCGTGTCCGGTCTGCGCGC     1440
Sheep_NCBI_New      CCGGTTGAGAGCGGCGGAGAGGCCAAGCACCCGGGACGCTTCCGTGTCCGGTCTGCGCGC     1089


Sheep Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          CGAGCCGCGGAGCTGAAAGGACCCC-----------------------------------     1465
Sheep_NCBI_New      CGAGCCGCGGAGCTGAAAGGACCCCTTCAAGTGGGAGAGTCCCAGTTCCGCAGCTCCTGG     1149


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          ------------------------------------------------------------     1465
Sheep NCBI New      ACCGGAAAGTGGAATCAGGCTCGCGAGGCTCACAGACCCTCCGCACGACACGCCGCGAGC     1209


Sheep_Ensembl       ------------------------------------------------------------     0
Sheep_NCBI          ------------------------------------------------------------     1465
Sheep NCBI New      AGACCTGCAGCCAGTGGGTTTCCCGGAACCCTTAGATTCTTCTGTAAACAGTTTTGCCTT     1269


Sheep_Ensembl       ---TTCTTCCTCCAGGGAACCTTCTCTCTGATCATTGAAGCGCTCCACACAGATTCTCCG     57
Sheep_NCBI          ----------TTCAAGGAACCTTCTCTCTGATCATTGAAGCGCTCCACACAGATTCTCCG     1515
Sheep_NCBI_New      CTGTTCTTCCTCCAGGGAACCTTCTCTCTGATCATTGAAGCGCTCCACACAGATTCTCCG     1329
                              * ** **********************************************


Sheep Ensembl       GATGACCTTGCAACAGAGAACCCAGAAAGACTTATCAGCCGCCTGGCCACACAGAGGCAC     117
Sheep_NCBI          GATGACCTTGCAACAGAGAACCCAGAAAGACTTATCAGCCGCCTGGCCACACAGAGGCAC     1575
Sheep_NCBI_New      GATGACCTTGCAACAGAGAACCCAGAAAGACTTATCAGCCGCCTGGCCACACAGAGGCAC     1389
                    ************************************************************


Sheep Ensembl       CTGGCCGTTGGGGAGGAGTGGTCCCAGGATCTGCACAGCAGCGGCCGCACGGACCTCAAG     177
Sheep NCBI          CTGGCCGTTGGGGAGGAGTGGTCCCAGGATCTGCACAGCAGCGGCCGCACGGACCTCAAG     1635
Sheep_NCBI_New      CTGGCCGTTGGGGAGGAGTGGTCCCAGGATCTGCACAGCAGCGGCCGCACGGACCTCAAG     1449
                    ************************************************************


Sheep_Ensembl       TACTCCTACCGCTTTGTGTGTGATGAGCACTACTTTGGCGAAGGCTGCTCCGTCTTCTGC     237
Sheep_NCBI          TACTCCTACCGCTTTGTGTGTGATGAGCACTACTTTGGCGAAGGCTGCTCCGTCTTCTGC     1695
Sheep NCBI New      TACTCCTACCGCTTTGTGTGTGATGAGCACTACTTTGGCGAAGGCTGCTCCGTCTTCTGC     1509
                    ************************************************************


Sheep_Ensembl       CGCCCCCGAGATGACGCCTTTGGCCACTTCACCTGCGGGGAGAGAGGGGAGAAAGTCTGC     297
Sheep_NCBI          CGCCCCCGAGATGACGCCTTTGGCCACTTCACCTGCGGGGAGAGAGGGGAGAAAGTCTGC     1755
Sheep_NCBI_New      CGCCCCCGAGATGACGCCTTTGGCCACTTCACCTGCGGGGAGAGAGGGGAGAAAGTCTGC     1569
                    ************************************************************
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Sheep_Ensembl       AACCCTGGCTGGAAGGGCCAGTACTGCACAGAGCCCATCTGTTTGCCAGGGTGCGATGAG     357
Sheep_NCBI          AACCCTGGCTGGAAGGGCCAGTACTGCACAGAGCCCATCTGTTTGCCAGGGTGCGATGAG     1815
Sheep_NCBI_New      AACCCTGGCTGGAAGGGCCAGTACTGCACAGAGCCCATCTGTTTGCCAGGGTGCGATGAG     1629
                    ************************************************************

Sheep_Ensembl       CAGCACGGATTTTGTGACAAGCCAGGGGAATGCAAAGTCAGACCCTCCTGGGGAGGAAGG     417
Sheep_NCBI          CAGCACGGATTTTGTGACAAGCCAGGGGAATGCAAGG------------------------     1852
Sheep_NCBI_New      CAGCACGGATTTTGTGACAAGCCAGGGGAATGCAAGG------------------------     1666
                    ********************************** *

Sheep_Ensembl       AGCTGTCTGGCCCGTCTCGGCGCCCGGTCCCCCCGGTCCCCAGGCTGTCCCCGCGCCCCC     477
Sheep NCBI          ----------CCCGTCTCGGCGCCCGGTCCCCCCGGTCCCCAGGCTGTCCCCGCGCCCCC     1902
Sheep NCBI New      ----------CCCGTCTCGGCGCCCGGTCCCCCCGGTCCCCAGGCTGTCCCCGCGCCCCC     1716
                             **************************************************

Sheep_Ensembl       TGCCAGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGGGGGGGGCCTCTTCTGCAACCAG     537
Sheep NCBI          TGCCAGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGGGGGGGGCCTCTTCTGCAACCAG     1962
Sheep NCBI New      TGCCAGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGGGGGGGGCCTCTTCTGCAACCAG     1776
                    ************************************************************

Sheep_Ensembl       GACCTCAACTACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACG     597
Sheep_NCBI          GACCTCAACTACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACG     2022
Sheep_NCBI_New      GACCTCAACTACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACG     1836
                    ************************************************************

Sheep Ensembl       GGCCAAGGGAGCTACACGTGCTGGTCTGAGGCAGGACTGGGAGGGTTCTTGGGAGCGACT     657
Sheep_NCBI          GGCCAAGGGAGCTACACGTGCTGGTCTGAGGCAGGACTGGGAGGGTTCTTGGGAGCGACT     2082
Sheep_NCBI_New      GGCCAAGGGAGCTACACGTGCTGGTCTGAGGCAGGACTGGGAGGGTTCTTGGGAGCGACT     1896
                    ************************************************************

Sheep Ensembl       AGGAAAGCTTTCCTCGTCTTCTCCGTCACTCAGGACCTTGAGAACAGCTACTCCTGCACC     717
Sheep_NCBI          AGGAAAGCTTTCCTCGTCTTCTCCGTCACTCAGGACCTTGAGAACAGCTACTCCTGCACC     2142
Sheep_NCBI_New      AGGAAAGCTTTCCTCGTCTTCTCCGTCACTCAGGACCTTGAGAACAGCTACTCCTGCACC     1956
                    ************************************************************

Sheep_Ensembl       TGCCCGCCTGGCTTCTACGGCAGGGTCTGCGAGCTGAGTGCCATGGTGTGTGCCGACGGC     777
Sheep NCBI          TGCCCGCCTGGCTTCTACGGCAGGGTCTGCGAGCTGAGTGCCATGGTGTGTGCCGACGGC     2202
Sheep NCBI New      TGCCCGCCTGGCTTCTACGGCAGGGTCTGCGAGCTGAGTGCCATGGTGTGTGCCGACGGC     2016
                    ************************************************************

Sheep_Ensembl       CCCTGCTTCAATGGGGGCCGGTGCTCCGACAACCCCGAGGGCGGGTACACCTGCCGCTGT     837
Sheep NCBI          CCCTGCTTCAATGGGGGCCGGTGCTCCGACAACCCCGAGGGCGGGTACACCTGCCGCTGT     2262
Sheep NCBI New      CCCTGCTTCAATGGGGGCCGGTGCTCCGACAACCCCGAGGGCGGGTACACCTGCCGCTGT     2076
                    ************************************************************

Sheep_Ensembl       CCTGGGGGCTTTTCTGGCTTTAACTGTGAGAAGAAGATGGATTCCTGCAGTTCCTCGCCT     897
Sheep_NCBI          CCTGGGGGCTTTTCTGGCTTTAACTGTGAGAAGAAGATGGATTCCTGCAGTTCCTCGCCT     2322
Sheep_NCBI_New      CCTGGGGGCTTTTCTGGCTTTAACTGTGAGAAGAAGATGGATTCCTGCAGTTCCTCGCCT     2136
                    ************************************************************

Sheep_Ensembl       TGTTCCAATGGTGCGCAGTGCGTGGACCTCGGCGACGCTTACGTCTGCCGCTGCCAGGCT     957
Sheep_NCBI          TGTTCCAATGGTGCGCAGTGCGTGGACCTCGGCGACGCTTACGTCTGCCGCTGCCAGGCT     2382
Sheep_NCBI_New      TGTTCCAATGGTGCGCAGTGCGTGGACCTCGGCGACGCTTACGTCTGCCGCTGCCAGGCT     2196
                    ************************************************************

Sheep Ensembl       GGCTTCTCTGGGAGGCACTGTGACGACAACGTGGACGACTGTGCCTCCTCCCCGTGTGCC     1017
Sheep NCBI          GGCTTCTCTGGGAGGCACTGTGACGACAACGTGGACGACTGTGCCTCCTCCCCGTGTGCC     2442
Sheep_NCBI_New      GGCTTCTCTGGGAGGCACTGTGACGACAACGTGGACGACTGTGCCTCCTCCCCGTGTGCC     2256
                    ************************************************************

Sheep_Ensembl       CACGGGGGCACCTGCCGGGACGGCGTGAACGAGTACTCCTGCACCTGCCCCCCCGGGTAC     1077
Sheep_NCBI          CACGGGGGCACCTGCCGGGACGGCGTGAACGAGTACTCCTGCACCTGCCCCCCCGGGTAC     2502
Sheep NCBI New      CACGGGGGCACCTGCCGGGACGGCGTGAACGAGTACTCCTGCACCTGCCCCCCCGGGTAC     2316
                    ************************************************************

Sheep_Ensembl       ACGGGCAGGAACTGCAGTGCCCCCATCAGCAGGTGCGAGCACGCGCCCTGCCACAACGGG     1137
Sheep_NCBI          ACGGGCAGGAACTGCAGTGCCCCCATCAGCAGGTGCGAGCACGCGCCCTGCCACAACGGG     2562
Sheep NCBI New      ACGGGCAGGAACTGCAGTGCCCCCATCAGCAGGTGCGAGCACGCGCCCTGCCACAACGGG     2376
                    ************************************************************

Sheep_Ensembl       GCCACCTGCCACGAACGGGCCTTCCGCTACCTGTGCGAGTGTGCCCGGGGCTACGGGCCC     1197
Sheep_NCBI          GCCACCTGCCACGAACGGGCCTTCCGCTACCTGTGCGAGTGTGCCCGGGGCTACGGGGGA     2622
Sheep_NCBI_New      GCCACCTGCCACGAACGGGCCTTCCGCTACCTGTGCGAGTGTGCCCGGGGCTACGGGGGA     2436
                    ************************************************************

Sheep Ensembl       ACACCGCCCGCCACAGGTCTCTGCGCGGGAGGAGACGGGCCCCCCGACACTCAGAGGGTG     1257
Sheep_NCBI          CAGGCTGGGAAGGGGGGCCTTCCCGGGGAGGGCGCCCCGACACCCGGGG-----------     2671
Sheep_NCBI_New      CAGGCTGGGAAGGGGGGCCTTCCCGGGGAGGGCGCCCCGACACCCGGGG-----------     2485
                        *          ** **   ** **  ** * *  * * ****

Sheep Ensembl       AGTGCTTCGGCCTGGAGGGGGCACATGGGGAGGCCAGCTGGTCAGGGGGCCAGCTGGAGA     1317
Sheep NCBI          ------------------------------------------------------------     2671
Sheep_NCBI_New      ------------------------------------------------------------     2485
```

```
Sheep_Ensembl       AGGTGCCCCGACACTCAAGGAATGGGCAGAACAAGCTGGGCAGGGGGGCCAGCTGGAGAG     1377
Sheep_NCBI          ------------------------------------------------------------     2671
Sheep NCBI New      ------------------------------------------------------------     2485

Sheep_Ensembl       GGCACCCCGACACCCGAGGGATGGGCAGGACAGGCTGGGAAGGGGGGCCTTCCCGGGGAG     1437
Sheep_NCBI          ------------------------------------------------------------     2671
Sheep_NCBI_New      ------------------------------------------------------------     2485

Sheep Ensembl       GGCGCCCCGACACCCGGGGGCCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCC     1497
Sheep NCBI          ------------------GCCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCC     2712
Sheep_NCBI_New      ------------------GCCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCC     2526
                                      ****************************************

Sheep Ensembl       ACGCAGATCAAGAACACCAACAAGAAGGCAGACTTCCACGTGGAGCCCCGCGGGAGGAGA     1557
Sheep NCBI          ACGCAGATCAAGAACACCAACAAGAAGGCAGACTTCCACGTGGAGCCCCGCGGGAGGAGA     2772
Sheep_NCBI_New      ACGCAGATCAAGAACACCAACAAGAAGGCAGACTTCCACGTGGAGCCCCGCGGGAGGAGA     2586
                    ************************************************************

Sheep_Ensembl       CGGGCCCCCCGACACTCAGAGGGTGAGTGCTTCGGCCTGGAGGGGGCACATGGGGAGGCC     1617
Sheep_NCBI          CGGGCCCCCCGACACTCAGAGGGTGAGTGCTTCGGCCTGGAGGGGGCACATGGGGAGGCC     2832
Sheep NCBI New      CGGGCCCCCCGACACTCAGAGGAATTTT------------------------------CC     2616
                    **********************     *                              **

Sheep_Ensembl       AGCTGGTCAGGGGGCCAGCTGGAGAAGGTGCCCCGACACTCAAGGAATGGGCAGAACAAG     1677
Sheep_NCBI          AGCTGTGGAGAAGC-----------------------------------------------     2846
Sheep NCBI New      TCTAGTGGAGAAGC-----------------------------------------------     2630
                        *   **  *

Sheep_Ensembl       CTGGGCAGGGGGGCCAGCTGGAGAGGGCACCCCGACACCCGAGGGATTGGAGAAGCGTCT     1737
Sheep_NCBI          ------------------------------------------------------GTCT     2850
Sheep_NCBI_New      ------------------------------------------------------GTCT     2634
                                                                           ****

Sheep Ensembl       GAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAAAGTACCAGTCGGTG     1797
Sheep NCBI          GAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAAAGTACCAGTCGGTG     2910
Sheep_NCBI_New      GAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAAAGTACCAGTCGGTG     2694
                    ************************************************************

Sheep Ensembl       TACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGGTC------------     1845
Sheep NCBI          TACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGGTCAGAGCGGGCTTG     2970
Sheep_NCBI_New      TACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGGTGTGA---------     2745
                    *********************************************

Sheep_Ensembl       -------------------------------------------------    1845
Sheep_NCBI          CGGGGCCCGCCTGCAGCTGGGGAGCCAGAACTGGGCCTCCGGCCGCGCTAA   3021
Sheep_NCBI_New      -------------------------------------------------    2745
```

Sheep and Cattle *DLL1* Sequence Alignment

```
Sheep_NCBI      ATGGACCCACACTCCCAACCACTGCAGATCCTGCCGGCCCTGGGCTGGCCTGGGAAGCTG          60
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      TGCGTGTTTCTGATGCAGAAGACGGACTTCACAGAACCCCCTCAACCGCAGGCCAGGGTG          120
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GCGCCGGGGGGAGCAGGCAGGGCGCACAGCTGTGGGGTCGAGCGGTCAGGGAGACGCGGT          180
Cattle_NCBI     ------------------------------------------------------------          0

Sheep NCBI      TCAGGGACCGCGACGGCGCCGAATCGCGAGTGCACCCTCAGCGCCGAGGCGACAGCCCCG          240
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      CAGGCCCAGCCCTACCTAGAGACGCGCCAGGACACCGGAGACGCGCCCACGCCGCCCTCG          300
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GCCCTCAGCAGCTGTGCGCACTTCCGCTTCCCGTCGCGAGACCGCCCCGCCCGCGCGGGG          360
Cattle_NCBI     ------------------------------------------------------------          0

Sheep NCBI      GCGGGGCCTGCGCGGCCGGCCGGGAGGCGCCGGGAGCGCCACGCGCAGGCGCCTGAGAGC          420
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GCGTGGCGGTGCCGGCTCCGGCTCTGCGCCCGTTGCCGCGGAAACCGCGCTGTGCTTCCG          480
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      AGGTCGGGGTCACGACTCGCTCGGCTTCCGCAGGCGTACCCGTTGGTGCTGTGGGCGGCG          540
Cattle_NCBI     ------------------------------------------------------------          0

Sheep NCBI      CAGCGGCGGTGGGGCCCCGCCTCGGTGCAGGTGACCGCGTGCGCCCTGTGCCGCGCGCCC          600
Cattle NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GGCGACCAGGGCTGGCCCGTCGACCTCGCCATCCCAGCCCGCGCGCATGTCCCTGCGACG          660
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GGTGCCCGCCGCCGCCACCCACTCCAAGCCACCCTCTGGAGCGCCCACCGGCGCCGCACC          720
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      CCCTCCAGGCAGCCCTCCGGAGCGCCTGGCCCGGCGCGTTCAGTCGGGGGCCGCTCGGGC          780
Cattle NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GGGAGCGCGGAGCAGCTGCGCGGTCATCTGATCCGGACAAAGGCGCCCGGAGCCGCGCCA          840
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      GTGAAAGTCACGTTTTGCCAGGACAGTGCAGTCCTGACAGAGAGGCCGGCTGCAGCACAC          900
Cattle_NCBI     ------------------------------------------------------------          0

Sheep_NCBI      CTACAGCGAGGCCTCAGTGACCGGGCGCCCTGTGCGGTACTGCTCATCAACGTGACCTTT          960
Cattle NCBI     ------------------------------------------------------------          0

Sheep_NCBI      ACTCCACGCCCACACCTTTCACCCCGGATGACGGTGCCCCGGGTGTTGGGGGTGGGGGAG          1020
Cattle_NCBI     ------------------------------------------------------------          0

Sheep NCBI      ATGGAAGGAGGAGCAGATAGGGGTGAGATAGAGACCTCAACCTATGCGGGCGGCCGGGAC          1080
Cattle_NCBI     ----------------------------------------ATGGGCGGGC---------          10
                                                         *  ******

Sheep_NCBI      CGCCGCCCCCGAGTGAGCCGGCCGGGCCGATCCTGCTCCACCGCCCCGGATTACCG----          1136
Cattle_NCBI     ------------------GGTGCGCCCTGACCCTCGCCGTACTCTCGGCCCTGCTGTGCC          52
                                  *  **  * ** ***   *   * * * *   * * *

Sheep_NCBI      ---CCTCGCGCGCGGACCTAAGGAACCTGGGCCTCCGGAAGAGCCGGAGCCGGACCCTTC          1193
Cattle_NCBI     AGGTCTGGAGCTCCGGGGTGTTCGAGCTGAAGCTGCAGGAGTTCGTCAACAAGAAGGGGC          112
                    ** * ** * *   *     * ***   ** * * **  *   * *  **     *
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```
Sheep_NCBI      AGGAGAGAAGTCCGCATTATCTCGGCCCGAGGCAGAGCGGCGGCGCTTTCCTGCCCGAGC          1253
Cattle_NCBI     TGCTGGGGAACC-GCA---ACTGCTGCCGCGGGGGCGCGGGGCCGCCGCCGTGC------          162
                 *  * * *  * ***    **    *** **  * **** * ***   * ***

Sheep_NCBI      TCCCGGGGACACCTTCCTCCTCCAGCGTCCCCTGACGACTTCGGAAGACAAGGCGGGGGG          1313
Cattle_NCBI     -GCCTGCAGGACCTTCTTCCGCGTGTG---CCTCA------AGCACTACCAGGCCAGCGT          212
                  ** *    ****** *** *  * *   *** *       * *  ** ****  * *

Sheep_NCBI      GCCCTCCAAGCCCGGGTGGCTCACAGCCGGCGGCAGCGGCGAGGGGGTGTTGCGCGGGGT          1373
Cattle_NCBI     GTCCCCCGAGCCGCCCTGCACCTAC---GGCAGCGCCGTCACCCCGGTGCTGGGCACCGA          269
                * ** ** ****    **   *      *** **  ** *     **** ** **   *

Sheep_NCBI      GACAGCCCCGGTTGAGAGCGGCGGAGAGGCCAAGCACCCGGGACGCTTCCGTGTCCGGTC          1433
Cattle_NCBI     CTCCTTCAGCCTCCCGGACGGCGCGGGCGCGGACC---------------CCGCCTTCAG          314
                  *   *    *   *  *****  *  **  * *                 * *

Sheep NCBI      TGCGCGCCGAGCCGCGGAGCTGAAAGGACCCCTTCAAGGAACCTTCTCTCTGATCATTGA          1493
Cattle_NCBI     CAACCCCATCCGCTTCCCCTTTGGCTTCACCTGGCCGGGAACCTTCTCTCTGATCATTGA          374
                    * *     *       *        **   *  **********************

Sheep_NCBI      AGCGCTCCACACAGATTCTCCGGATGACCTTGCAACAGAGAACCCAGAAAGACTTATCAG          1553
Cattle_NCBI     AGCTCTCCACACAGATTCTCCTGATGACCTTGCAACAGAGAACCCAGAAAGACTTATCAG          434
                *** ***************** ***************************************

Sheep NCBI      CCGCCTGGCCACACAGAGGCACCTGGCCGTTGGGGAGGAGTGGTCCCAGGATCTGCACAG          1613
Cattle_NCBI     CCGCCTGGCCACACAGAGGCACCTGGCGGTTGGGGAGGAGTGGTCCCAGGACCTGCACAG          494
                ************************** ********************** ********

Sheep NCBI      CAGCGGCCGCACGGACCTCAAGTACTCCTACCGCTTTGTGTGTGATGAGCACTACTTTGG          1673
Cattle NCBI     CAGCGGCCGCACAGACCTCAAGTACTCCTACCGCTTTGTGTGTGATGAGCACTACTTCGG          554
                ************ ********************************************** **

Sheep_NCBI      CGAAGGCTGCTCCGTCTTCTGCCGCCCCCCGAGATGACGCCTTTGGCCACTTCACCTGCGG          1733
Cattle_NCBI     GGAAGGCTGCTCCGTCTTCTGCCGCCCCCCGAGATGACGCCTTTGGACACTTCACCTGCGG          614
                 ******************************************** ***************

Sheep NCBI      GGAGAGAGGGGAGAAAGTCTGCAACCCTGGCTGGAAGGGCCAGTACTGCACAGAGCCCAT          1793
Cattle_NCBI     GGAGAGAGGGGAGAAAGTCTGCAACCCCGGCTGGAAGGGCCAGTACTGCACAGAACCCAT          674
                ************************** ************************** *****

Sheep NCBI      CTGTTTGCCAGGGTGCGATGAGCAGCACGGATTTTGTGACAAGCCAGGGGAATGCAAGGC          1853
Cattle NCBI     CTGTTTGCCAGGGTGCGATGAGCAGCACGGATTTTGTGACAAGCCAGGGGAATGCAAGTG          734
                ************************************************************

Sheep_NCBI      CCGTCTCGGCG------------------CCCGGTCCCCCCGGTCCCCAGGCTGTCCCCG          1895
Cattle_NCBI     CAGAGTGGGCTGGCAGGGCCGGTACTGTGACCAGTGCATTCGGTACCCAGGCTGTCTCCA          794
                * *  * ***                    ** ** *   **** ********** **

Sheep_NCBI      CGCCCCCTGCCAGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGGGGGGGGCCTCTTCTG          1955
Cattle_NCBI     CGGCACCTGCCGGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGGGGGGGGCCTTTTCTG          854
                ** * ****** ********************************************* *****

Sheep_NCBI      CAACCAGGACCTCAACTACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCAC          2015
Cattle NCBI     CAACCAGGACCTCAACTACTGCACGCACCATAAGCCCTGCAGGAACGGGGCCACCTGCAC          914
                ******************************** ***************** ************

Sheep_NCBI      CAACACGGGCCAAGGGAGCTACACGTGCTGGTCTGAGGCAGGACTGGGAGGGTTCTTGGG          2075
Cattle_NCBI     CAACACGGGCCAAGGGAGCTACACATGCTCGTGCCGGCCTGGGTACACGGGGGCCAACTG          974
                ************************ **** **    * * **       ***  *    *

Sheep_NCBI      AGCGAC------------------TAGGAAAGCTTTCCTCGTCTTCTCCGTCACTCAGGA          2117
Cattle NCBI     TGAGACGGAGGTGGACGAGTGCAGCGCGGGGCCTTGCAGGAACGGAGGGAGCTGCACGGA          1034
                 * ***                    *    *** *     *        *     ***

Sheep_NCBI      CCTTGAGAACAGCTACTCCTGCACCTGCCCGCCTGGCTTCTACGGCAGGGTCTGCGAGCT          2177
Cattle_NCBI     CCTTGAGAACAGCTACTCCTGCACCTGCCCACCCGGCTTCTACGGCAGGATCTGCGAGCT          1094
                ******************************** ** *************** **********

Sheep_NCBI      GAGTGCCATGGTGTGTGCCGACGGCCCCTGCTTCAATGGGGGCCGGTGCTCCGACAACCC          2237
Cattle_NCBI     GAGTGCCATGGTGTGTGCCGACGGTCCCTGCTTCAACGGGGGCCGGTGCTCCGACAACCC          1154
                ************************ *********** ************************

Sheep_NCBI      CGAGGGCGGGTACACCTGCCGCTGTCCTGGGGGCTTTTCTGGCTTTAACTGTGAGAAGAA          2297
Cattle NCBI     CGAGGGCGGGTACACCTGCCGCTGCCCTGGGGGCTTCTCCGGCTTTAACTGTGAGAAGAA          1214
                ************************ *********** ** ********************

Sheep_NCBI      GATGGATTCCTGCAGTTCCTCGCCTTGTTCCAATGGTGCGCAGTGCGTGGACCTCGGCGA          2357
Cattle_NCBI     GACGGATTCCTGCAGCTCCTCGCCTTGTTCCAATGGTGCTCAATGCGTGGACCTCGGCGA          1274
                ** ************ ********************** ** *****************

Sheep NCBI      CGCTTACGTCTGCCGCTGCCAGGCTGGCTTCTCTGGGAGGCACTGTGACGACAACGTGGA          2417
Cattle_NCBI     CGCTTACGCCTGCCGCTGCCAGGCCGGCTTCTCTGGGAGGCACTGTGACAACAACGTGGA          1334
                ******** *************** ************************ **********
```

```
Sheep_NCBI      CGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAACGAGTA        2477
Cattle_NCBI     CGACTGTGCCTCCTCCCCATGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAACGAGTA        1394
                ****************** ****************************************

Sheep_NCBI      CTCCTGCACCTGCCCCCCCGGGGTACACGGGCAGGAACTGCAGTGCCCCCATCAGCAGGTG        2537
Cattle_NCBI     CTCCTGCACCTGCCCCCCCGGGGTACACGGGCAGGAACTGCAGTGCCCCCGTCAGCAGGTG        1454
                ********************************************** **********

Sheep_NCBI      CGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTACCTGTG        2597
Cattle_NCBI     CGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCATGAGCGGGCCTTCCGCTACCTGTG        1514
                ********************************** ** ********************

Sheep_NCBI      CGAGTGTGCCCGGGGCTACGGGGGACAGGCTGGGAAGGGGGGCCTTCCCGGGGAGGGCGC        2657
Cattle_NCBI     TGAGTGCGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAGCCGCCCCC        1574
                 ***** ***************** *      *  **      ** **** *  *  * *

Sheep NCBI      CCCGACACCCGGGGG---CCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCCAC        2714
Cattle_NCBI     GGGCCCCGTGGTGGTGGACCTCACAGAGAAGTACGTGGAGGGCCAGGCCG------GCCC        1628
                     *    * **    **     ****** ****    * *   * *        * *

Sheep_NCBI      GCAGATCAAGAACACCAACAAGAAGGCAGACTTCCACGTGGAGC-CCCGCGGGAGGAGAC        2773
Cattle_NCBI     GTTCCCCTGGGTGGCCGTCGGGGCGGGCGTGCTGCTGGTCCTCACGCTGCTGCTGGGCTG        1688
                *     *  *    **  *  *  **  *   * *  **       * ** *  **

Sheep NCBI      GGGCCCCCCGACACTCAGAGGGTGAGTGCTTCG--GCCTGGAGGGGGCACATGGGGAGGC        2831
Cattle_NCBI     CGCCGCCACGGTGGTCTGCGTGCGGCTGAGGCTGCAGAAGCGCCGGCCCCCTGCAGACCC        1748
                 * * ** **    ** * * * *  **   *       *    ** * * **  **  *

Sheep NCBI      CAGCTGTGGA--------------------------GAAGCGTCTGAAAGAAAAAGGC--        2863
Cattle NCBI     CTGCCGGGGAGAGACAGAGACCATGAACAACCTGGCCAACCGCCAGCGGGAGAAGGACAT        1808
                * ** * ***                           ** ** * *   ** ** * *

Sheep_NCBI      CGGACTCTGTGTACTCTGCTTCA--------AAAGACACAAAGTACCAGTCGGTG---TA        2912
Cattle_NCBI     CTCTGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGACTTCCA        1868
                *    ** * **  ** *   **        **  **** **  *  ** * *      *

Sheep NCBI      CGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGGTCAGAGCGGGCTTGCG        2972
Cattle_NCBI     CGTGGAGCCCGGCGCGGAGAAAAACGGCCTCACGGCCCGAGA----------CTCTGCCG        1918
                ***     ***  * * ** *  *  *  ***  **    **           **  **

Sheep NCBI      GGGCCCGCCTGCAGCTGGGGAGCCAGAACTGGGCCTCCGGCCGCGCTAA-----------        3021
Cattle NCBI     TGGGCTGCAACCTGCTGCAGGGCCTTAAGGGCGCCGCAGCCACGGCGGGGCCCCACAGCG        1978
                 ** * **   * ****  * ***  **  * *** * * *   **

Sheep_NCBI      ------------------------------------------------------------        3021
Cattle_NCBI     TGCGCGATGCCAAGGGCCAGCCCCAAGGCTCTGCAGGGGAGGAGAAGGGCACCCCGACAC        2038

Sheep NCBI      ------------------------------------------------------------        3021
Cattle_NCBI     TCAGAGGTGGAGAAGCGTCTGAAAGAAAAAGGCCAGACTCTGTGTACTCTGCTTCAAAAG        2098

Sheep_NCBI      ------------------------------------------------------------        3021
Cattle NCBI     ACACAAAGTACCAGTCTGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCCA        2158

Sheep_NCBI      -----------    3021
Cattle_NCBI     CTGAGGTGTGA    2169
```

***DLL1* sequence "a"**

```
  1    ACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACGGGCCAAGGGA  60
 61    GCTACACGTGCTCCTGCCGGCCTGGGTACACGGGGGCCGGCTGCGAGACGGAGGTGGACG  120
121    AGTGCCGCGGCGGGCCTTGCAGGAACGGCGGGAGCTGCACGGTGAGTCCAGCCGCCGCGG  180
181    GCCGTCCCGTCGGCCGGGAGCCCTGGTCTGAGGCAGACTGGGAGGGTTCTTGGGAGCGAC  240
241    TAGGAAAGCTTTCCTCGTCTTCTCCGTCACTCAGGACC                        278
```

***DLL1* sequence "a" alignment against Sheep *DLL1* (XM_004011569.1)**

```
Query  1     ACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACGGGCCAAGGGA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1973  ACTGCACGCACCACAAGCCCTGCAGGAACGGCGCCACCTGCACCAACACGGGCCAAGGGA  2032

Query  61    GCTACACGTGCT  72
             ||||||||||||
Sbjct  2033  GCTACACGTGCT  2044

Query  203   CTGGTCTGAGGCA-GACTGGGAGGGTTCTTGGGAGCGACTAGGAAAGCTTTCCTCGTCTT  261
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2043  CTGGTCTGAGGCAGGACTGGGAGGGTTCTTGGGAGCGACTAGGAAAGCTTTCCTCGTCTT  2102

Query  262   CTCCGTCACTCAGGACC  278
             |||||||||||||||||
Sbjct  2103  CTCCGTCACTCAGGACC  2119
```

Query = sequence "a". Subject = Sheep *DLL1* sequence

***DLL1* sequence "b"**

```
  1     CAGAAAGACTTATCAGCCGCCTGGCCACACAGAGGCACCTGGCCGTTGGGGAGGAGTGGT  60
 61     CCCAGGATCTGCACAGCAGCGGCCGCACGGACCTCAAGTACTCCTACCGCTTTGTGTGTG  120
121     ATGAGCACTACTTTGGCGAAGGCTGCTCCGTCTTCTGCCGCCCCCGAGATGACGCCTTTG  180
181     GCCACTTCACCTGCGGGGAGAGAGGGGAGAAAGTCTGCA                       219
```

Sequence matches 100% to nucleotides 1538 – 1756 of XM_004011569.1

***DLL1* sequence "c"**

```
  1     CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
 61     CGAGTACTCCTGCACCTGCCCCCCCGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAG  120
121     CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  180
181     CCTGTGCGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAGAC  240
241     CCCTGCCGGGGGGGAGACGGAGACCATGAACAACCTGGCCAGCCGCCGGCGCGAGAAGGAC  300
301     GTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGAGAC       356
```

***DLL1* sequence "c" alignment against Sheep *DLL1* (XM_004011569.1)**

```
Query  1     CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
             || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2412  CGTGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  2471

Query  61    CGAGTACTCCTGCACCTGccccccccGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAG  120
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  2472  CGAGTACTCCTGCACCTGCCCCCCCCGGGTACACGGGCAGGAACTGCAGTGCCCCCATCAG  2531

Query  121   CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2532  CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  2591

Query  181   CCTGTGCGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAGAC  240
             ||||||||||||||||||||||||||||||     || | | |            | |
Sbjct  2592  CCTGTGCGAGTGTGCCCGGGGCTACGGGGG-----ACAGGCTG-------GGAAGGGGGG  2639

Query  241   CCCTGCCGGGGGGAGACGGAGACCATGAACAACCTGGCCAGCCGCCGGCGCGAGAAGGAC  300
             || | |||||    || || | ||    ||| || ||      ||||||||||||||||
Sbjct  2640  CCTTCCCGGG----GAGGGCGCCC--CGACACCCGGG-----GGCCGGCGCGAGAAGGAC  2688

Query  301   GTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGG-AGAC  356
             |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  2689  GTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGAC  2745
```

Query = sequence "c". Subject = Sheep *DLL1* sequence

***DLL1* sequence "c" alignment against Cattle *DLL1* (XM_010808803.1)**

```
Query  1     CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
             || ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1848  CGTGGACGACTGTGCCTCCTCCCCATGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  1907

Query  61    CGAGTACTCCTGCACCTGcccccccGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAG  120
             |||||||||||||||||||||||||||||||||||||||| |||||||||||||| ||||
Sbjct  1908  CGAGTACTCCTGCACCTGCCCCCCCCGGGTACACGGGCAGGAACTGCAGTGCCCCCGTCAG  1967

Query  121   CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  180
             |||||||||||||||||||||||||||||||||||||||| || ||||||||||||||
Sbjct  1968  CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCATGAGCGGGCCTTCCGCTA  2027

Query  181   CCTGTGCGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAG  238
             |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2028  CCTGTGTGAGTGCGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAG  2085

Query  237   AGACCCCTGCCGGGGGGAGACGGAGACCATGAACAACCTGGCCAGCCGCCGGCGCGAGAA  296
             |||||||||||||| ||||| |||||||||||||||||||| ||||| ||| |||||
Sbjct  2262  AGACCCCTGCCGGGGAGAGACAGAGACCATGAACAACCTGGCCAACCGCCAGCGGGAGAA  2321

Query  297   GGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGG-AGA  355
             |||| |||| ||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  2322  GGACATCTCTGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGA  2381

Query  356   C  356
             |
Sbjct  2382  C  2382
```

Query = sequence "c". Subject = Cattle *DLL1* sequence

DLL1 sequence "d" (3' RACE)

```
  1    AGGGCGCCCCGACACTCAGAGGTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGT  60
 61    ACTCTGCTTCAAAAGACACAAAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACG  120
121    AGTGTGTCATCGCAACTGAGGTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAG  180
181    AACAAGGAACTTCCGAGGTGTCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCC  240
241    TGACTGCTGCTGGAAGCCAGGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCG  300
301    CTGCTCTGCCTCTAGGACGTTCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATA  360
361    TTTAAATGAATGGACTTGATCACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCT  420
421    GGATTCTTATGAGCCAGTCTTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCT  480
481    TTTTGATACTAAAATGTGTTTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGT  540
541    AAAAATATTTTTCATGATATCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAA  600
601    TTTAAATTTTTGGTAAATATGTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTG  660
661    TATATAAATGTATTTATGGAATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTT  720
721    AATGAAGAAATTCCTTTTTAAAATATTTTTCCAAAATAAATATTATGAACAACA        774
```

DLL1 sequence "d" alignment against Goat _DLL1_ (XM_005685019.1)

```
Query  21    GGTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACA  80
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1345  GGTGGAGAAGCATCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACA  1404

Query  81    AAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAG  140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1405  AAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAG  1464

Query  141   GTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGTG  200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1465  GTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGTG  1524

Query  201   TCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCAG  260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1525  TCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCAG  1584

Query  261   GTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCTGCTCTGCCTCTAGGACGT  320
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1585  GTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCCGCTCTGCCTCTAGGACGT  1644
```

```
Query  321   TCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATATTTAAATGAATGGACTTGAT  380
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1645  TCCCATTGCACTATGGACGGTTGCTTTTAAAAAATATATATTTAAATGAATGGACTTGAT  1704

Query  381   CACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGTCT  440
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1705  CACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTTTGGATTCTTATGAGCCAGTCT  1764

Query  441   TCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGTT  500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1765  TCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGTT  1824

Query  501   TTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATAT  560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1825  TTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATAT  1884

Query  561   CTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATAT  620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1885  CTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATAT  1944

Query  621   GTACAAAGGCACTTCGGGTCAATGTGACTATAttttttttGTATATAAATGTATTTATGGA  680
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1945  GTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTTGTATATAAATGTATTTATGGA  2004

Query  681   ATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTTAATGAAGAAATTCCTTTTTA  740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2005  ATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTTAATGAAGAAATTCCTTTTTA  2064

Query  741   AAATATTTTTCCAAAATAAATATTATGAACAACA  774
             ||||||||||||||||||||||||||||||||||
Sbjct  2065  AAATATTTTTCCAAAATAAATATTATGAACAACA  2098
```

Query = sequence “c”. Subject = Goat *DLL1* sequence.

DLL1 sequence "d" alignment against Cattle _DLL1_ (XM_010808803.1)

```
Query  1     AGGGCGCCCCGACACTCAGAGGTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGT  60
             ||||| ||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  2543  AGGGCACCCCGACACTCAGAGGTGGAGAAGCGTCTGAAAGAAAAAGGCCAGACTCTGTGT  2602

Query  61    ACTCTGCTTCAAAAGACACAAAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACG  120
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  2603  ACTCTGCTTCAAAAGACACAAAGTACCAGTCTGTGTACGTCATATCCGAGGAGCAGGACG  2662

Query  121   AGTGTGTCATCGCAACTGAGGTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAG  180
             |||||||||||||| |||||||||||||| |||||||||||||||||||||||||| ||||||
Sbjct  2663  AGTGTGTCATCGCCACTGAGGTGTGACATGGAGGTGACGGGGCAAGTTCCGTTTCTGCAG  2722

Query  181   AACAAGGAACTTCCGAGGTGTCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCC  240
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  2723  AACAAGGAACTTCCGAGGTGTCG-CCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCC  2781

Query  241   TGACTGCTGCTGGAAGCCAGGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCG  300
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  2782  TGACTGCTGCTGGAAGCCAGGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCGGCCG  2841

Query  301   CTGCTCTGCCTCTAGGACGTTCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATA  360
             |||||||||||||||||||| |||||||||||||||| || ||||||||||||||||||
Sbjct  2842  CTGCTCTGCCTCTAGGACGTTTCCATTGCACTATGGACAGTTGCTTTTAAAAAATATATA  2901

Query  361   TTTAAATGAATGGACTTGATCACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCT  420
             |||||| ||||||||| |||||||| |||||||||||||| |||||||||||||| ||||||
Sbjct  2902  TTTAAACGAATGGACTCGATCACTAGGTAAGAAGCACGCGCTGCCTGAAGTGTGTATTCT  2961

Query  421   GGATTCTTATGAGCCAGTCTTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCT  480
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  2962  GGATTCTTATGAGCCAGTCCTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCT  3021

Query  481   TTTTGATACTAAAATGTGTTTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGT  540
             ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3022  TTTTGATACAAAAATGTGTTTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGT  3081

Query  541   AAAAATATTTTTCATGATATCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3082  AAAAATATTTTTCATGATATCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAA  3141
```

```
Query  601   TTTAAATTTTTGGTAAATATGTACAAAGGCACTTCGGGTCAATGTGACTATAttttttttG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3142  TTTAAATTTTTGGTAAATATGTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTG  3201

Query  661   TATATAAATGTATTTATGGAATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3202  TATATAAATGTATTTATGGAATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTT  3261

Query  721   AATGAAGAAATTCCTTTTTAAAATATTTTTCCAAAATAAATATTATGAACAACA  774
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3262  AATGAAGAAATTCCTTTTTAAAATATTTTTCCAAAATAAATATTATGAACAACA  3315
```

Query = sequence “c”. Subject = Cattle *DLL1* sequence.

***DLL1* sequence “e”**

```
  1   CGCAGATCAAGAACACCAACAAGAAGGCAGACTTCCACGTGGAGCCCAGTGCGGAGAAAA  60
 61   ATGGCCTCACAGCTCGAGACCCCGCCGTGGGCTACAACCTGCTGCAGGGCCTCAAGGGCA  120
121   CCTCAGCCACTGCGGAGCCCCATGGCAAGCACGATGCCAAGTGCCAGCCCCAGGGCTCTG  180
181   CGGGGGAGGAGAAGGGCGCCCCGACACTCAGAGGTGGAGAAGCGTCTGAAA           231
```

DLL1 combined sequence (sequences "c" through "e")

```
   1   CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
  61   CGAGTACTCCTGCACCTGCCCCCCCGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAG  120
 121   CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  180
 181   CCTGTGCGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAGAC  240
 241   CCCTGCCGGGGGGAGACGGAGACCATGAACAACCTGGCCAGCCGCCGGCGCGAGAAGGAC  300
 301   GTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGACTTC  360
 361   CACGTAGAGCCCAGTGCGGAGAAAAATGGCCTCACAGCTCGAGACCCCGCCGTGGGCTAC  420
 421   AACCTGCTGCAGGGCCTCAAGGGCACCTCAGCCACTGCGGAGCCCCATGGCAAGCACGAT  480
 481   GCCAAGTGCCAGCCCCAGGGCTCTGCGGGGGAGGAGAAGGGCGCCCCGACACTCAGAGGT  540
 541   GGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAAAG  600
 601   TACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGGTG  660
 661   TGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGTGTCG  720
 721   GCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCAGGTC  780
 781   CTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCTGCTCTGCCTCTAGGACGTTCC  840
 841   CATTGCACTATGGACGGTCGCTTTTAAAAAATATATATTTAAATGAATGGACTTGATCAC  900
 901   TACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGTCTTCT  960
 961   CTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGTTTTT  1020
1021   CTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATATCTG  1080
1081   TAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATATGTA  1140
1141   CAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTGTATATAAAT               1186
```

***DLL1* combined sequence alignment against Cattle *DLL1* (XM_010808803.1)**

```
Query  1    CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
            || ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  198  CGTGGACGACTGTGCCTCCTCCCCATGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  257

Query  61   CGAGTACTCCTGCACCTGcccccccGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAG  120
            |||||||||||||||||||||||||||||||||||||||| |||||||||||||| ||||
Sbjct  258  CGAGTACTCCTGCACCTGCCCCCCCCGGGTACACGGGCAGGAACTGCAGTGCCCCCGTCAG  317

Query  121  CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTA  180
            |||||||||||||||||||||||||||||||||||||||| || ||||||||||||||
Sbjct  318  CAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCATGAGCGGGCCTTCCGCTA  377

Query  181  CCTGTGCGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAG  238
            |||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  378  CCTGTGTGAGTGCGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTCCCCGAG  435


Query  237   AGACCCCTGCCGGGGGGAGACGGAGACCATGAACAACCTGGCCAGCCGCCGGCGCGAGAA  296
            ||||||||||||||| ||||| |||||||||||||||||||| ||||| ||| |||||
Sbjct  612   AGACCCCTGCCGGGGAGAGACAGAGACCATGAACAACCTGGCCAACCGCCAGCGGGAGAA  671

Query  297   GGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGA  356
            |||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  672   GGACATCTCTGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCAGA  731

Query  357   CTTCCACGTAGAGCCCAGTGCGGAGAAAAATGGCCTCACAGCTCGAGACCCCGCCGTGGG  416
            |||||||| |||||| | |||||||||| |||||||| || |||||| | ||||||||
Sbjct  732   CTTCCACGTGGAGCCCGGCGCGGAGAAAAACGGCCTCACGGCCCGAGACTCTGCCGTGGG  791

Query  417   CTACAACCTGCTGCAGGGCCTCAAGGGCACCTCAGCCACTGCGGAGCCCCATGGCAAGCA  476
            || |||||||||||||||| |||||| || ||||||| |||| ||||||  ||  ||
Sbjct  792   CTGCAACCTGCTGCAGGGCCTTAAGGGCGCCGCAGCCACGGCGGGGCCCCACAGCGTGCG  851

Query  477   CGATGCCAAGTGCCAGCCCCAGGGCTCTGCGGGGGAGGAGAAGGGCGCCCCGACACTCAG  536
            |||||||||| |||||||||| |||||||| ||||||||||||||| ||||||||||||
Sbjct  852   CGATGCCAAGGGCCAGCCCCAAGGCTCTGCAGGGGAGGAGAAGGGCACCCCGACACTCAG  911

Query  537   AGGTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACAC  596
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  912   AGGTGGAGAAGCGTCTGAAAGAAAAAGGCCAGACTCTGTGTACTCTGCTTCAAAAGACAC  971
```

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Query  597   AAAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGA  656
              |||||||||||| ||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  972   AAAGTACCAGTCTGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCCACTGA  1031

Query  657   GGTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGT  716
              |||||||| ||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  1032  GGTGTGACATGGAGGTGACGGGGCAAGTTCCGTTTCTGCAGAACAAGGAACTTCCGAGGT  1091

Query  717   GTCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCA  776
              |||| | ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1092  GTCG-CTCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCA  1150

Query  777   GGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCTGCTCTGCCTCTAGGACG  836
              |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  1151  GGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCGGCCGCTGCTCTGCCTCTAGGACG  1210

Query  837   TTCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATATTTAAATGAATGGACTTGA  896
              || ||||||||||||||| || |||||||||||||||||||||| ||||||||| ||
Sbjct  1211  TTTCCATTGCACTATGGACAGTTGCTTTTAAAAAATATATATTTAAACGAATGGACTCGA  1270

Query  897   TCACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGTC  956
              |||||| |||||||||||||| |||||||||||||| ||||||||||||||||||||||||
Sbjct  1271  TCACTAGGTAAGAAGCACGCGCTGCCTGAAGTGTGTATTCTGGATTCTTATGAGCCAGTC  1330

Query  957   TTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGT  1016
               ||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  1331  CTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACAAAAATGTGT  1390

Query  1017  TTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATA  1076
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1391  TTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATA  1450

Query  1077  TCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATA  1136
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1451  TCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATA  1510

Query  1137  TGTACAAAGGCACTTCGGGTCAATGTGACTATAttttttttGTATATAAAT  1186
              |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1511  TGTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTTGTATATAAAT  1560
```

Query = *DLL1* combined sequence. Subject = Cattle *DLL1*

DLL1 combined sequence alignment against Goat _DLL1_ (XM_005685019.1)

```
Query  1     CGAGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAA  60
             || ||||||||||||||||||||||||||||||||||||||||| |||||| |||||||||
Sbjct  1098  CGTGGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCCGCCGGGGCGGCGTGAA  1157

Query  61    CGAGTACTCCTGCACCTGcccccccGGGTACACGGGC  97
             |||| ||||| ||||| |||||||| ||  |||| ||
Sbjct  1158  CGAGGACTCCCGCACCCGCCCCCCCCGGGCCACGCGC  1194


Query  288   GCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAA  347
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1191  GCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAA  1250

Query  348   GAAGGCAGACTTCCACGTAGAGCCCAGTGCGGAGAAAAATGGCCTCACAGCTCGAGACCC  407
             |||||||||||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1251  GAAGGCAGACTTCCATGTGGAGCCCAGTGCGGAGAAAAATGGCCTCACAGCTCGAGACCC  1310

Query  408   CGCCGTGGGCTACAACCTGCTGCAGGG  434
             |||||||||||||||||||||||||||
Sbjct  1311  CGCCGTGGGCTACAACCTGCTGCAGGG  1337


Query  538   GGTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACA  597
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1345  GGTGGAGAAGCATCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACA  1404

Query  598   AAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAG  657
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1405  AAGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAG  1464

Query  658   GTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGTG  717
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1465  GTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGGTG  1524

Query  718   TCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCAG  777
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1525  TCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCCAG  1584

Query  778   GTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCTGCTCTGCCTCTAGGACGT  837
             |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1585  GTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCCGCTCTGCCTCTAGGACGT  1644
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```
Query  838   TCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATATTTAAATGAATGGACTTGAT  897
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1645  TCCCATTGCACTATGGACGGTTGCTTTTAAAAAATATATATTTAAATGAATGGACTTGAT  1704

Query  898   CACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGTCT  957
             |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  1705  CACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTTTGGATTCTTATGAGCCAGTCT  1764

Query  958   TCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGTT  1017
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1765  TCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTGTT  1824

Query  1018  TTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATAT  1077
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1825  TTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGATAT  1884

Query  1078  CTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATAT  1137
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1885  CTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAATAT  1944

Query  1138  GTACAAAGGCACTTCGGGTCAATGTGACTATAttttttttGTATATAAAT  1186
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1945  GTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTTGTATATAAAT  1993
```

Query = *DLL1* combined sequence. Subject = Goat *DLL1*

DLL1 combined sequence alignment against Sheep genome OAR v3.1

```
Query  4         GGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAACGA  63
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90488244  GGACGACTGTGCCTCCTCCCCGTGTGCCCACGGGGGCACCTGCCGGGACGGCGTGAACGA  90488185

Query  64        GTACTCCTGCACCTGcccccccGGGTACACGGGCAGGAGCTGCAGTGCCCCCATCAGCAG  123
                 |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  90488184  GTACTCCTGCACCTGCCCCCCCGGGTACACGGGCAGGAACTGCAGTGCCCCCATCAGCAG  90488125

Query  124       GTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTACCT  183
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90488124  GTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCACGAACGGGCCTTCCGCTACCT  90488065

Query  184       GTGCGAGTGTGCCCGGGGCTACGGGG  209
                 |||||||||||||||||||||||||||
Sbjct  90488064  GTGCGAGTGTGCCCGGGGCTACGGGG  90488039

Query  284       GCCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCA  343
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90487040  GCCGGCGCGAGAAGGACGTCTCCGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCA  90486981

Query  344       ACAAGAAGGCAGACTTCCACGTAGAGCCC  372
                 |||||||||||||||||||||| ||||||
Sbjct  90486980  ACAAGAAGGCAGACTTCCACGTGGAGCCC  90486952

Query  539       GTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAA  598
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486464  GTGGAGAAGCGTCTGAAAGAAAAAGGCCGGACTCTGTGTACTCTGCTTCAAAAGACACAA  90486405

Query  599       AGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGG  658
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486404  AGTACCAGTCGGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATCGCAACTGAGG  90486345

Query  659       T  659
                 |
Sbjct  90486344  T  90486344

Query  656       AGGTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGG  715
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486255  AGGTGTGACGTGGAGGTGACGGGGCAAGTTCCGTTCCTGCAGAACAAGGAACTTCCGAGG  90486196

Query  716       TGTCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCC  775
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486195  TGTCGGCCCCGACGATGCTGCTGGGAGGAGCGGACGTGCTCCTGACTGCTGCTGGAAGCC  90486136
```

```
Query  776       AGGTCCTCTTCCTGCCGGGGGCGGCGGGCGCTCCCGCAGCCGCTGCTCTGCCTCTAGGAC  835
                       | |  || || ||||| || |||||| ||||||||||||||||||||||||||||
Sbjct  90486135  -----C-C--CCGGCGGGGGGGGGGGGGCGCCCCCGCAGCCGCTGCTCTGCCTCTAGGAC  90486084

Query  836       GTTCCCATTGCACTATGGACGGtcgcttttaaaaaatatatatttaaatgaatggaCTTG  895
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486083  GTTCCCATTGCACTATGGACGGTCGCTTTTAAAAAATATATATTTAAATGAATGGACTTG  90486024

Query  896       ATCACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGT  955
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90486023  ATCACTACGTAAGAAGCACGCACTGCCTGAAGTGTATATTCTGGATTCTTATGAGCCAGT  90485964

Query  956       CTTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGAtactaaaatgtg  1015
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90485963  CTTCTCTTGAATTAGAATCACAAACACTGCCTTTATTGTCCTTTTTGATACTAAAATGTG  90485904

Query  1016      tttttctagGTGGAAAAAATGTGCGTTATTTTTTGgacttgtaaaaatatttttcatgat  1075
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90485903  TTTTTCTAGGTGGAAAAAATGTGCGTTATTTTTTGGACTTGTAAAAATATTTTTCATGAT  90485844

Query  1076      atCTGTAAAGCTTGAGTATTTTGTGAtgttcatttttataatttaaatttttggtaAAT  1135
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90485843  ATCTGTAAAGCTTGAGTATTTTGTGATGTTCATTTTTTATAATTTAAATTTTTGGTAAAT  90485784

Query  1136      ATGTACAAAGGCACTTCGGGtcaatgtgactatatttttttgtatataaatgtatttatg  1195
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90485783  ATGTACAAAGGCACTTCGGGTCAATGTGACTATATTTTTTTGTATATAAATGTATTTATG  90485724

Query  1196      gaaTATTGTGCAAATGTTATTTGagtttttttactgttttgttaatgaagaaattccttttt  1255
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  90485723  GAATATTGTGCAAATGTTATTTGAGTTTTTTACTGTTTTGTTAATGAAGAAATTCCTTTT  90485664

Query  1256      taaaatatttttccaaaataaatattatgaacaacA  1291
                  |||||||||||||||||||||||||||||||||||
Sbjct  90485663  TAAAATATTTTTCCAAAATAAATATTATGAACAACA  90485628
```

Query = *DLL1* combined sequence. Subject = Sheep genome OAR v3.1

DLL1 is predicted to be encoded on chromosome 8, nucleotides 90523290 – 90486280.

Cattle, Pig, Human and Mouse Alignment of *DLL1* sequences

```
Cattle_DLL1      ATGGGCGGGCGGTGCGCCCTGACCCTCGCCGTACTCTCGGCCCTGCTGTGCCAGGTCTGG 60
Pig_DLL1         ATGGGCCGGCGGTGCGCCCTGGCCCTCGCCGTGCTTTCGGCCCTGCTGTGCCAGGTCTGG 60
Human_DLL1       ATGGGCAGTCGGTGCGCGCTGGCCCTGGCGGTGCTCTCGGCCTTGCTGTGTCAGGTCTGG 60
Mouse_DLL1       ATGGGCCGTCGGAGCGCGCTAGCCCTTGCCGTGGTCTCTGCCCTGCTGTGCCAGGTCTGG 60
                 ****** * ***:**** **..**** ** **. * ** *** ******* *********

Cattle_DLL1      AGCTCCGGGGTGTTCGAGCTGAAGCTGCAGGAGTTCGTCAACAAGAAGGGGCTGCTGGGG 120
Pig_DLL1         AGCTCCGGGGTGTTCGAGCTGAAGCTGCAGGAGTTCGTCAACAAGAAGGGGCTGCTGGGA 120
Human_DLL1       AGCTCTGGGGTGTTCGAACTGAAGCTGCAGGAGTTCGTCAACAAGAAGGGGCTGCTGGGG 120
Mouse_DLL1       AGCTCCGGCGTATTTGAGCTGAAGCTGCAGGAGTTCGTCAACAAGAAGGGGCTGCTGGGG 120
                 ***** ** **.** **.****************************************.

Cattle_DLL1      AACCGCAACTGCTGCCGCGGGGGCGCGGGGCCGCCGCCGTGCGCCTGCAGGACCTTCTTC 180
Pig_DLL1         AACCGCAACTGCTGCCGCGGAGGCGCGGGGCCGCCGCCGTGCGCCTGCAGGACCTTCTTC 180
Human_DLL1       AACCGCAACTGCTGCCGCGGGGGCGCGGGGCCACCGCCGTGCGCCTGCCGGACCTTCTTC 180
Mouse_DLL1       AACCGCAACTGCTGCCGCGGGGGCTCTGG---CCCGCCTTGCGCCTGCAGGACCTTCTTT 177
                 ******************.*** * **    ***** *********.**********

Cattle_DLL1      CGCGTGTGCCTCAAGCACTACCAGGCCAGCGTGTCCCCCGAGCCGCCCTGCACCTACGGC 240
Pig_DLL1         CGCGTGTGCCTCAAGCACTACCAGGCCAGCGTGTCTCCCGAGCCGCCCTGCACCTACGGC 240
Human_DLL1       CGCGTGTGCCTCAAGCACTACCAGGCCAGCGTGTCCCCCGAGCCGCCCTGCACCTACGGC 240
Mouse_DLL1       CGCGTATGCCTCAAGCACTACCAGGCCAGCGTGTCACCGGAGCCACCCTGCACCTACGGC 237
                 *****.***************************** ** *****.***************

Cattle_DLL1      AGCGCCGTCACCCCGGTGCTGGGCACCGACTCCTTCAGCCTCCCGGACGGCGCGGGCGCG 300
Pig_DLL1         AGCGCTGTCACGCCGGTGCTGGGCGTCGACTCCTTCAGCCTCCCTGACGGCGCGGGCGCC 300
Human_DLL1       AGCGCCGTCACCCCCGTGCTGGGCGTCGACTCCTTCAGTCTGCCCGACGGCGGGGGCGCC 300
Mouse_DLL1       AGTGCTGTCACGCCAGTGCTGGGTGTCGACTCCTTCAGCCTGCCTGATGGCGCAGGCATC 297
                 ** ** ***** ** ******** . ************ ** ** ** **** .***.

Cattle_DLL1      GACCCCGCCTTCAGCAACCCCATCCGCTTCCCCTTTGGCTTCACCTGGCCGGGAACCTTC 360
Pig_DLL1         GACCCCGCCTTCAGCAACCCCATCCGCTTCCCCTTTGGCTTCACCTGGCCGGGAACTTTC 360
Human_DLL1       GACTCCGCGTTCAGCAACCCCATCCGCTTCCCCTTCGGCTTCACCTGGCCGGGCACCTTC 360
Mouse_DLL1       GACCCCGCCTTCAGCAACCCCATCCGATTCCCCTTCGGCTTCACCTGGCCAGGTACCTTC 357
                 *** **** *****************.******** **************.** ** ***

Cattle_DLL1      TCTCTGATCATTGAAGCTCTCCACACAGATTCTCCTGATGACCTTGCAACAGAGAACCCA 420
Pig_DLL1         TCTCTGATCATTGAAGCTCTGCACACGGATTCTCCCGATGACCTCGCAACAGAAAACCCA 420
Human_DLL1       TCTCTGATTATTGAAGCTCTCCACACAGATTCTCCTGATGACCTCGCAACAGAAAACCCA 420
Mouse_DLL1       TCTCTGATCATTGAAGCCCTCCATACAGACTCTCCCGATGACCTCGCAACAGAAAACCCA 417
                 ******** ******** ** ** **.** ***** ******** ********.******

Cattle_DLL1      GAAAGACTTATCAGCCGCCTGGCCACACAGAGGCACCTGGCGGTTGGGGAGGAGTGGTCC 480
Pig_DLL1         GAGAGACTCATCAGCCGCCTGGCCACACAGAGGCACCTCACCGTGGGCGAGGAGTGGTCC 480
Human_DLL1       GAAAGACTCATCAGCCGCCTGGCCACCCAGAGGCACCTGACGGTGGGCGAGGAGTGGTCC 480
Mouse_DLL1       GAAAGACTCATCAGCCGCCTGACCACACAGAGGCACCTCACTGTGGGAGAAGAATGGTCT 477
                 **.***** ************.****.*********** .* ** ** **.**.*****

Cattle_DLL1      CAGGACCTGCACAGCAGCGGCCGCACAGACCTCAAGTACTCCTACCGCTTTGTGTGTGAT 540
Pig_DLL1         CAGGACCTGCACAGCAGTGGCCGCACGGACCTCAAGTATTCCTACCGCTTCGTGTGTGAC 540
Human_DLL1       CAGGACCTGCACAGCAGCGGCCGCACGGACCTCAAGTACTCCTACCGCTTCGTGTGTGAC 540
Mouse_DLL1       CAGGACCTTCACAGTAGCGGCCGCACAGACCTCCGGTACTCTTACCGGTTTGTGTGTGAC 537
                 ******** ***** ** ********.******..*** ** ***** ** ********

Cattle_DLL1      GAGCACTACTTCGGGGAAGGCTGCTCCGTCTTCTGCCGCCCCCGAGATGACGCCTTTGGA 600
Pig_DLL1         GAGCACTACTATGGGGAGGGCTGCTCTGTCTTCTGCCGCCCCCGAGACGACGCCTTTGGC 600
Human_DLL1       GAACACTACTACGGAGAGGGCTGCTCCGTTTTCTGCCGTCCCCGGGACGATGCCTTCGGC 600
Mouse_DLL1       GAGCACTACTACGGAGAAGGTTGCTCTGTGTTCTGCCGACCTCGGGATGACGCCTTTGGC 597
                 **.*******: **.**.** ***** ** ******** ** **.** ** ***** **.

Cattle_DLL1      CACTTCACCTGCGGGGAGAGAGGGGAGAAAGTCTGCAACCCCGGCTGGAAGGGCCAGTAC 660
Pig_DLL1         CACTTCACCTGCGGGGAGAGAGGCGAGAAAGTCTGCAACCCTGGCTGGAAAGGCCAGTAC 660
Human_DLL1       CACTTCACCTGTGGGGAGCGTGGGGAGAAAGTGTGCAACCCTGGCTGGAAAGGGCCCTAC 660
Mouse_DLL1       CACTTCACCTGCGGGGACAGAGGGGAGAAGATGTGCGACCCTGGCTGGAAAGGCCAGTAC 657
                 *********** ***** .*:** *****..* ***.**** ********.** *. ***
```

```
Cattle_DLL1      TGCACAGAACCCATCTGTTTGCCAGGGTGCGATGAGCAGCACGGATTTTGTGACAAGCCA 720
Pig_DLL1         TGCACTGAGCCCATCTGTTTGCCGGGGTGCGACGAGCAGCATGGGTTTTGTGACAAGCCA 720
Human_DLL1       TGCACAGAGCCGATCTGCCTGCCTGGATGTGATGAGCAGCATGGATTTTGTGACAAACCA 720
Mouse_DLL1       TGCACTGACCCAATCTGTCTGCCAGGGTGTGATGACCAACATGGATACTGTGACAAACCA 717
                 *****:** ** *****  **** **.** ** ** **.** **.*: ********.***

Cattle_DLL1      GGGGAATGCAAGTGCAGAGTGGGCTGGCAGGGCCGGTACTGTGACCAGTGCATTCGGTAC 780
Pig_DLL1         GGGGAATGCAAGTGCAGAGTGGGCTGGCAGGGCCGGTACTGTGACCAGTGCATTCGGTAC 780
Human_DLL1       GGGGAATGCAAGTGCAGAGTGGGCTGGCAGGGCCGGTACTGTGACGAGTGTATCCGCTAT 780
Mouse_DLL1       GGGGAGTGCAAGTGCAGAGTTGGCTGGCAGGGCCGCTACTGCGATGAGTGCATCCGATAC 777
                 *****.************** ************** ***** **  **** ** ** **

Cattle_DLL1      CCAGGCTGTCTCCACGGCACCTGCCGGCAGCCCTGGCAATGCAACTGCCAGGAGGGCTGG 840
Pig_DLL1         CCAGGCTGTCTCCACGGCACCTGCCAGCAGCCCTGGCAATGCAACTGCCAGGAAGGCTGG 840
Human_DLL1       CCAGGCTGTCTCCATGGCACCTGCCAGCAGCCCTGGCAGTGCAACTGCCAGGAAGGCTGG 840
Mouse_DLL1       CCAGGTTGTCTCCATGGCACCTGCCAGCAACCCTGGCAGTGTAACTGCCAGGAAGGCTGG 837
                 ***** ******** **********.***.********.** ***********.******

Cattle_DLL1      GGGGGCCTTTTCTGCAACCAGGACCTCAACTACTGCACGCACCATAAGCCCTGCAGGAAC 900
Pig_DLL1         GGGGGCCTTTTCTGCAACCAGGATCTGAACTACTGCACACACCACAAGCCCTGCAAGAAC 900
Human_DLL1       GGGGGCCTTTTCTGCAACCAGGACCTGAACTACTGCACACACCATAAGCCCTGCAAGAAT 900
Mouse_DLL1       GGGGGCCTTTTCTGCAACCAAGACCTGAACTACTGTACTCACCATAAGCCGTGCAGGAAT 897
                 ********************.** ** ******** ** ***** ***** ****.***

Cattle_DLL1      GGGGCCACCTGCACCAACACGGGCCAAGGGAGCTACACATGCTCGTGCCGGCCTGGGTAC 960
Pig_DLL1         GGGGCCACCTGCACCAATACGGGCCAGGGTAGCTACACTTGCTCTTGCCGGCCGGGGTAC 960
Human_DLL1       GGAGCCACCTGCACCAACACGGGCCAGGGGAGCTACACTTGCTCTTGCCGGCCTGGGTAC 960
Mouse_DLL1       GGAGCCACCTGCACCAACACGGGCCAGGGGAGCTACACATGTTCCTGCCGACCTGGGTAT 957
                 **.************** ********.** ********:** ** *****.** *****

Cattle_DLL1      ACGGGGGCCAACTGTGAGACGGAGGTGGACGAGTGCAGCGCGGGGCCTTGCAGGAACGGA 1020
Pig_DLL1         ACGGGGGCCAACTGTGAGACAGAGGTGGACGAGTGCAAGGCCAGCCCCTGCAGGAATGGA 1020
Human_DLL1       ACAGGTGCCACCTGCGAGCTGGGGATTGACGAGTGTGACCCCAGCCCTTGTAAGAACGGA 1020
Mouse_DLL1       ACAGGTGCCAACTGTGAGCTGGAAGTAGATGAGTGTGCTCCTAGCCCCTGCAAGAACGGA 1017
                 **.** ****.*** ***. .*...* ** ***** .   * .* ** ** *.*** ***

Cattle_DLL1      GGGAGCTGCACGGACCTTGAGAACAGCTACTCCTGCACCTGCCCACCCGGCTTCTACGGC 1080
Pig_DLL1         GGGAGCTGCACGGACCTCGAGAACAGCTTCTCCTGCACCTGCCCACCTGGCTTCTATGGC 1080
Human_DLL1       GGGAGCTGCACGGATCTCGAGAACAGCTACTCCTGTACCTGCCCACCCGGCTTCTACGGC 1080
Mouse_DLL1       GCGAGCTGCACGGACCTTGAGGACAGCTTCTCTTGCACCTGCCCTCCCGGCTTCTATGGC 1077
                 * ************ ** ***.******:*** ** ********:** ******** ***

Cattle_DLL1      AGGATCTGCGAGCTGAGTGCCATGGTGTGTGCCGACGGTCCCTGCTTCAACGGGGGCCGG 1140
Pig_DLL1         AGGATCTGCGAGCTGAGTGCTATGGCGTGTGCGGACGGCCCTTGCTTCAACGGGGGCCGG 1140
Human_DLL1       AAAATCTGTGAATTGAGTGCCATGACCTGTGCGGACGGCCCTTGCTTTAACGGGGGTCGG 1140
Mouse_DLL1       AAGGTCTGTGAGCTGAGCGCCATGACCTGTGCAGATGGCCCTTGCTTCAATGGAGGACGA 1137
                 *...**** **. **** ** ***.  ***** ** ** ** ***** ** **.** **.

Cattle_DLL1      TGCTCCGACAACCCCGAGGGCGGGTACACCTGCCGCTGCCCTGGGGGCTTCTCCGGCTTT 1200
Pig_DLL1         TGCTCTGACAACCCCGAGGGAGGGTACACCTGCCGCTGCCCTGGGGGCTTCTCTGGCTTT 1200
Human_DLL1       TGCTCAGACAGCCCCGATGGAGGGTACAGCTGCCGCTGCCCCGTGGGCTACTCCGGCTTC 1200
Mouse_DLL1       TGTTCAGATAACCCTGACGGAGGCTACACCTGCCATTGCCCCTTGGGCTTCTCTGGCTTC 1197
                 ** ** ** *.*** ** **.** **** *****. *****   *****:*** *****

Cattle_DLL1      AACTGTGAGAAGAAGACGGATTCCTGCAGCTCCTCGCCTTGTTCCAATGGTGCTCAATGC 1260
Pig_DLL1         AACTGTGAGAAGAAGATGGATTCCTGCACTTCCTCACCCTGTTCCAATGGTGCGCAGTGT 1260
Human_DLL1       AACTGTGAGAAGAAAATTGACTACTGCAGCTCTTCACCCTGTTCTAATGGTGCCAAGTGT 1260
Mouse_DLL1       AACTGTGAGAAGAAGATGGATCTCTGCGGCTCTTCCCCTTGTTCTAACGGTGCCAAGTGT 1257
                 **************.*  **   ****.  ** ** ** ***** ** ***** .*.**

Cattle_DLL1      GTGGACCTCGGCGACGCTTACGCCTGCCGCTGCCAGGCCGGCTTCTCTGGGAGGCACTGT 1320
Pig_DLL1         GTGGACCTCGGCGATGCCTACCTCTGCCGCTGCCGGGCTGGCTTCTCTGGGAGGCACTGC 1320
Human_DLL1       GTGGACCTCGGTGATGCCTACCTGTGCCGCTGCCAGGCCGGCTTCTCGGGGAGGCACTGT 1320
Mouse_DLL1       GTGGACCTCGGCAACTCTTACCTGTGCCGGTGCCAGGCTGGCTTCTCCGGGAGGTACTGC 1317
                 *********** .*  * ***   ***** ****.*** ******** ****** ****

Cattle_DLL1      GACAACAACGTGGACGACTGTGCCTCCTCCCCATGTGCCCACGGGGGCACCTGCCGGGAC 1380
Pig_DLL1         GAGGACAACGTGGACGACTGTGCCTCCTCCCCGTGTGCCAATGGAGGCGCCTGCAGGGAC 1380
Human_DLL1       GACGACAACGTGGACGACTGCGCCTCCTCCCCGTGCGCCAACGGGGGCACCTGCCGGGAT 1380
Mouse_DLL1       GAGGACAATGTGGATGACTGTGCCTCCTCCCCGTGTGCAAATGGGGGCACCTGCCGGGAC 1377
                 ** .**** ***** ***** ***********.** **..* **.***.*****.****
```

```
Cattle_DLL1     GGCGTGAACGAGTACTCCTGCACCTGCCCCCCCGGGTACACGGGCAGGAACTGCAGTGCC 1440
Pig DLL1        GGTGTGAACGAGTACTCCTGCACCTGCCCCCCCGGGTACACGGGCAGGAACTGCAGCGCC 1440
Human DLL1      GGCGTGAACGACTTCTCCTGCACCTGCCCGCCTGGCTACACGGGCAGGAACTGCAGTGCC 1440
Mouse_DLL1      AGTGTGAACGACTTCTCCTGTACCTGCCCACCTGGCTACACGGGCAAGAACTGCAGCGCC 1437
                .* ******** *:****** ******** ** ** **********.********* ***

Cattle_DLL1     CCCGTCAGCAGGTGCGAGCACGCGCCCTGCCACAACGGGGCCACCTGCCATGAGCGGGCC 1500
Pig DLL1        CCCGTCAGTAGGTGTGAGCATTCGCCCTGCCACAACGGGGCCACCTGCCACGAGAGGGCC 1500
Human_DLL1      CCCGTCAGCAGGTGCGAGCACGCACCCTGCCACAATGGGGCCACCTGCCACGAGAGGGGC 1500
Mouse_DLL1      CCTGTCAGCAGGTGTGAGCATGCACCCTGCCATAATGGGGCCACCTGCCACCAGAGGGGC 1497
                ** ***** ***** *****  *.******** ** **********  **.*** *

Cattle_DLL1     TTCCGCTACCTGTGTGAGTGCGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTC 1560
Pig DLL1        CTCCGCTACCTGTGTGAGTGTGCCCGGGGCTACGGGGGCCCCAACTGCCAGTTCCTGCTT 1560
Human_DLL1      CACCGCTATGTGTGCGAGTGTGCCCGAGGCTACGGGGGTCCCAACTGCCAGTTCCTGCTC 1560
Mouse_DLL1      CAGCGCTACATGTGTGAGTGCGCCCAGGGCTATGGCGGCCCCAACTGCCAGTTTCTGCTC 1557
                 : *****  **** ***** ****..***** ** ** ********** *****

Cattle_DLL1     CCCGAGCCGCCCCCGGGCCCCGTGGTGGTGGACCTCACAGAGAAGTACGTGGAGGGCCAG 1620
Pig_DLL1        CCTGCACCACCCCCGGGCCCCGTGGTGGTGGACCTCACCGAGAAGTACGTGGAGGGCCAG 1620
Human_DLL1      CCCGAGCTGCCCCCGGGCCCAGCGGTGGTGGACCTCACTGAGAAG---CTAGAGGGCCAG 1617
Mouse_DLL1      CCTGAGCCACCACCAGGGCCCATGGTGGTGGACCTCAGTGAGAGGCATATGGAGAGCCAG 1617
                ** *..* .**.**.** **.. **********  ****.*    *.***.*****

Cattle_DLL1     GCCGGCCCGTTCCCCTGGGTGGCCGTCGGGGCGGGCGTGCTGCTGGTCCTCACGCTGCTG 1680
Pig_DLL1        ACTGGGCCGTTCCCCTGGGTGGCCGTCTGTGCGGGCGTGGTTCTCGTCCTCATGCTGCTG 1680
Human_DLL1      GGCGGGCCATTCCCCTGGGTGGCCGTGTGCGCCGGGGTCATCCTTGTCCTCATGCTGCTG 1677
Mouse_DLL1      GGCGGGCCCTTCCCCTGGGTGGCCGTGTGTGCCGGGGTGGTGCTTGTCCTCCTGCTGCTG 1677
                .  ** ** *****************  * ** ** **  * ** ******. *******

Cattle_DLL1     CTGGGCTGCGCCGCCACGGTGGTCTGCGTGCGGCTGAGGCTGCAGAAGCGCCGGCCCCCT 1740
Pig_DLL1        CTCGGCTGTGCCGCTGTGGTGGTCTGCACGCGGCTAAGGCTGCAGAAGCGCCGGCCTCCC 1740
Human_DLL1      CTGGGCTGTGCCGCTGTGGTGGTCTGCGTCCGGCTGAGGCTGCAGAAGCACCGGCCCCCA 1737
Mouse_DLL1      CTGGGCTGTGCTGCTGTGGTGGTCTGCGTCCGGCTGAAGCTACAGAAACACCAGCCTCCA 1737
                ** ***** ** ** . **********.  *****.*.***.*****.*.**.*** **

Cattle_DLL1     GCAGACCCCTGCCGGGGAGAGACAGAGACCATGAACAACCTGGCCAACCGCCAGCGGGAG 1800
Pig_DLL1        GCCGACCCCTGCCGGGGGGAGACTGAGACCATGAACAACCTGGCCAACTGTCAGCGGGAG 1800
Human_DLL1      GCCGACCCCTGCCGGGGGGAGACGGAGACCATGAACAACCTGGCCAACTGCCAGCGTGAG 1797
Mouse_DLL1      CCTGAACCCTGTGGGGGAGAGACAGAAACCATGAACAACCTAGCCAATTGCCAGCGCGAG 1797
                 * **.*****  ****.***** **.***************.*****  * ***** ***

Cattle_DLL1     AAGGACATCTCTGTCAGCGTCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCA 1860
Pig_DLL1        AAGGACATCTCTGTCAGCGTCATTGGGGCCACGCAGATCAAGAACACCAACAAGAAGGTG 1860
Human_DLL1      AAGGACATCTCAGTCAGCATCATCGGGGCCACGCAGATCAAGAACACCAACAAGAAGGCG 1857
Mouse_DLL1      AAGGACGTTTCTGTTAGCATCATTGGGGCTACCCAGATCAAGAACACCAACAAGAAGGCG 1857
                ******.* **:** ***.**** ***** ** ************************ .

Cattle_DLL1     GACTTCCACGTGGAGCCCGGCGCGGAGAAAAACGGCCTCACGGCCCGAGACTCTGCCGTG 1920
Pig_DLL1        GACCTCCAGGCAGAGCCCGGCTCGGAGAAGAATGGCCTCAAGGCTCGAGATCCCGCCGTG 1920
Human_DLL1      GACTTCCACGGGGACCACAGCGCCGACAAGAATGGCTTCAAGGCCCGCTACCCAGCGGTG 1917
Mouse_DLL1      GACTTTCACGGGGACCATGGAGCCGAGAAGAGCAGCTTTAAGGTCCGATACCCCACTGTG 1917
                *** * ** * .** *. .*. * ** **.*. .** * *.**  **. *  * .* ***

Cattle_DLL1     GGCTGCAACCTGCTGCAGGGCCTTAAGGGCGCCG---CAGCCACGGCGGGGCCCCACAGC 1977
Pig_DLL1        GGCTATAACCTGCTGCAGGACCTCAAGGGTGCTG---CTGCCACCAGGGACCCACACAGC 1977
Human_DLL1      GACTATAACCTCGTGCAGGACCTCAAGGGTGACGACACCGCCGTCAGGGACGCGCACAGC 1977
Mouse_DLL1      GACTATAACCTCGTTCGAGACCTCAAGGGAGATGAAGCCACGGTCAGGGATACACACAGC 1977
                *.**. *****  * *..*.*** ***** *. *   * .* .  . **.  * ******

Cattle_DLL1     GTGCGCGATGCCAAGGGCCAGCCCCAAGGCTCTGCAGGGGAGGAGAAGGGCACCCCG--- 2034
Pig_DLL1        AAGCCCGATGCCAAGTGCCAGCCCCACGGCTCTGCGGGGGAGGAGAAGGGCACCCTG--- 2034
Human_DLL1      AAGCGTGACACCAAGTGCCAGCCCCAGGGCTCCTCAGGGGAGGAGAAGGGGACCCCGACC 2037
Mouse_DLL1      AAACGTGACACCAAGTGCCAGTCACAGAGCTCTGCAGGAGAAGAGAAGATCGCCCCA--- 2034
                .:.*  ** .***** ***** *.** .****  *.**.**.******.  .*** .

Cattle_DLL1     ACACTCAGAGGTGGAGAAGCGTCTGAAAGAAAAAGGCCAGACTCTGTGTACTCTGCTTCA 2094
Pig DLL1        ACACTCAGGGGTGGAGAAGCACCTGAAAGAAAGAGGCCGGACTCTGTGTACGCCGCTTCA 2094
Human_DLL1      ACACTCAGGGGTGGAGAAGCATCTGAAAGAAAAAGGCCGGACTCGGGCTGTTCAACTTCA 2097
Mouse_DLL1      ACACTTAGGGGTGGGGAGATTCCTGACAGAAAAAGGCCAGAGTCTGTCTACTCTACTTCA 2094
                ***** **.*****.**..   ****.*****.*****.** ** *  *.  * .*****
```

```
Cattle DLL1      AAAGACACAAAGTACCAGTCTGTGTACGTCATATCCGAGGAGCAGGACGAGTGTGTCATC 2154
Pig DLL1         AAGGACACCAAGTACCAGTCGGTGTACGTCATATCCGAGGAGAAGGACGAGTGCGTCATC 2154
Human_DLL1       AAAGACACCAAGTACCAGTCGGTGTACGTCATATCCGAGGAGAAGGATGAGTGCGTCATA 2157
Mouse_DLL1       AAGGACACCAAGTACCAGTCGGTGTATGTTCTGTCTGCAGAAAAGGATGAGTGTGTTATA 2154
                 **.*****.********** ***** ** .*.** *..**..**** ***** ** **.

Cattle DLL1      GCCACTGAGGTGTGA---------------------- 2169
Pig_DLL1         GCCACCGAGGGGAGACGTTGGCTCTTGTCCCAGTGA 2190
Human_DLL1       GCAACTGAGGTGTAA---------------------- 2172
Mouse_DLL1       GCGACTGAGGTGTAA---------------------- 2169
                 ** ** **** *:.*
```

Accession numbers for cattle, pig, human and mouse sequences are as follows: XM_010808803.1, XM_003353151.2, NM_005618.3, NM_007865.3

Appendix 4: RNAHybrid analysis of sheep and cattle MHC class II genes

RNAHybrid analysis of sheep MHC class II DQA and DRA genes used for flow cytometry

```
TARGET : Sheep_MHCII_DQA1
length: 1148
MIRNA  : 17-28
length: 22

mfe: -22.6 kcal/mol
p-value: undefined

position  872
target 5'  A    ACU     UAGAU        G 3'
            ACAG     UGC     GACCUAGA
            UGUC     ACG     UUGGAUCU
miRNA  3' GA    GUUUU                 5'

TARGET : Sheep_MHCII_DQA2.1
length: 1129
MIRNA  : 17-28
length: 22

mfe: -21.4 kcal/mol
p-value: undefined

position  844
target 5' U          GAGGACGUUCUAGA         A 3'
           UGCAG AAGA              CGACCUAGA
           AUGUC UUUU              GUUGGAUCU
miRNA  3' G     G    AC                      5'

TARGET : Sheep_MHCII_DQA2.2
length: 1117
MIRNA  : 17-28
length: 22

mfe: -24.5 kcal/mol
p-value: undefined

position  848
target 5'  A    A      GACGCACUAGA         A 3'
            GCAG AGAGUG           CGACCUAGA
            UGUC UUUUAC           GUUGGAUCU
miRNA  3' GA    G                          5'
```

```
TARGET : Sheep_MHCII_DQA1
length: 1148
MIRNA  : 17-17
length: 22

mfe: -22.7 kcal/mol
p-value: undefined

position  1043
target 5'   A       C  UUAU       C  3'
             UUGUUAC UA    GGGAUCC
             AGCAGUG AU    UCCUAGG
miRNA  3' GCA       A             AA 5'

TARGET : Sheep_MHCII_DQA1
length: 1148
MIRNA  : 17-17
length: 22

mfe: -19.8 kcal/mol
p-value: undefined

position  997
target 5'       A     U        C 3'
                 UACUU GGGAUUCU
                 GUGAA UCCUAGGA
miRNA  3' GCAAGCA     U        A 5'

TARGET : Sheep_MHCII_DRA
length: 1205
MIRNA  : 17-17
length: 22

mfe: -19.4 kcal/mol
p-value: undefined

position  1070
target 5'       C               U 3'
                 CAU   AGGAUCCUU
                 GUG   UCCUAGGAA
miRNA  3' GCAAGCA   AAU           5'

TARGET : Sheep_MHCII_DRA
length: 1205
MIRNA  : 17-6
length: 23

mfe: -23.3 kcal/mol
p-value: undefined

position  791
target 5' A    C    AACGGA  UU        G  3'
           CCUG AGGU      CU  GUUAGAGA
           GGAU UCCA      GA  CGAUUUUU
miRNA  3'      C            GG        AU 5'
```

```
TARGET : Sheep_MHCII_DQA2.2
length: 1117
MIRNA  : 17-6
length: 23

mfe: -23.6 kcal/mol
p-value: undefined

position  814
target 5' C      AGGGAA   G      CCAUCUA      C  3'
           CCUAGA      GGU CUCUGC       UGAGAG
           GGAUCU      CCA GAGGCG       AUUUUU
miRNA  3'                                   AU 5'

TARGET : Sheep_MHCII_DQA1
length: 1148
MIRNA  : 17-2
length: 22

mfe: -24.7 kcal/mol
p-value: undefined

position  941
target 5' C    C U     UUCUU       A  3'
           UCCU C CAUAC     CUCUGGG
           AGGA G GUAUG     GGGGCCC
miRNA  3' C    C U                 CA 5'

TARGET : Sheep_MHCII_DQA2.1
length: 1129
MIRNA  : 17-2
length: 22

mfe: -28.1 kcal/mol
p-value: undefined

position  1020
target 5' U     CA   GU     ACCAUGAGAUCA       A 3'
           GUUCU  GUA  UGCCU            CUGGGGU
           CAGGA  CGU  AUGGG            GGCCCCA
miRNA  3'            GU                       5'

TARGET : Sheep_MHCII_DQA2.2
length: 1117
MIRNA  : 17-2
length: 22

mfe: -28.1 kcal/mol
p-value: undefined

position  1022
target 5' U     CA   GU     ACCAUGAUAUCA       A 3'
           GUUCU  GUA  UGCCU            CUGGGGU
           CAGGA  CGU  AUGGG            GGCCCCA
miRNA  3'            GU                       5'
```

RNAHybrid analysis of sheep MHC class II *DQA* and *DRA* genes used for luciferase assay

Sheep *DQA1*:

```
MIRNA  : 17-21
length: 25

mfe: -24.0 kcal/mol
p-value: undefined

position  962
target 5'    U                    G 3'
              GGGAC     UUAAGGUGCU
              CUCUG     GGUUCCACGA
miRNA  3' UUGU     UAGUG          A 5'

MIRNA  : 17-14
length: 25

mfe: -20.3 kcal/mol
p-value: undefined

position  1109
target 5'   C     UA    A   AAGAAAU       U 3'
             UAAUA   UCC UGG       AAAUGCC
             GUUGU   AGG ACC       UUUACGG
miRNA  3' CUU     CCG                    U 5'
```

Cattle *DQA1*:

```
MIRNA  : 17-30
length: 21

mfe: -18.9 kcal/mol
p-value: undefined

position  1072
target 5' U   AU  CU    UAUUC       U 3'
           GGA  UG  ACGG     CACCCAA
           UCU  AC  UGUC     GUGGGUU
miRNA  3'               CUCU        U 5'
```

Sheep *DQB*:

```
MIRNA  : 17-20
length: 23

mfe: -19.8 kcal/mol
p-value: undefined

position  921
target 5' A       A     UAGUGACUC        U 3'
           GAU CAG UCCUA         UGAUACAG
           UUG GUU GGGAU         ACUAUGUC
miRNA  3' G   U   G                    U 5'

MIRNA  : 17-9
length: 25

mfe: -25.6 kcal/mol
p-value: undefined

position  1102
target 5' G     A UCU       AU        A 3'
           AUCAG G   UUCUUAG  UAACUCUG
           UGGUC C   AGGAAUC  AUUGAGAU
miRNA  3' A     C UU                  5'
```

Sheep *DRA1*:

```
MIRNA  : 17-25
length: 21

mfe: -19.5 kcal/mol
p-value: undefined

position  888
target 5' C   A ACA    UGCUU       U 3'
           UGA G   ACCA     CAGCAUU
           ACU C   UGGU     GUCGUAA
miRNA  3'     C G      UUC         C 5'

MIRNA  : 17-17
length: 22

mfe: -19.4 kcal/mol
p-value: undefined

position  1070
target 5'       C               U 3'
                 CAU   AGGAUCCUU
                 GUG   UCCUAGGAA
miRNA  3' GCAAGCA   AAU           5'
```

```
MIRNA  : 24-1
length: 26

mfe: -27.6 kcal/mol
p-value: undefined

position  962
target 5'  G       AA       UUAUUCCCUGUC        C 3'
            GU  UCU  UGUGUAG            UACUGCUC
            CA  AGA  ACACAUC            AUGACGAG
miRNA  3' UA  AC   CG                           5'
```

Cattle *DRA1*:

```
MIRNA  : 17-17
length: 22

mfe: -19.4 kcal/mol
p-value: undefined

position  1128
target 5'       C               U 3'
                 CAU   AGGAUCCUU
                 GUG   UCCUAGGAA
miRNA  3' GCAAGCA   AAU           5'

MIRNA  : 24-1
length: 26

mfe: -20.9 kcal/mol
p-value: undefined

position  1033
target 5'  A   A  CC    C           C 3'
            GUU UU  CUGU    UACUGCUC
            CAA AG  GACA    AUGACGAG
miRNA  3' UA   C  AC    CAUC          5'
```

Cattle *DRB*:

```
MIRNA  : 17-9
length: 25

mfe: -21.8 kcal/mol
p-value: undefined

position  889
target 5'  C    CUUCUUCACAGCAU    AGGUU    GCU   UGC       U 3'
            CCAG              GGAA     UCCU   UAG   UAACUCU
            GGUC              CCUU     AGGA   AUC   AUUGAGA
miRNA  3' AU                                            U 5'
```

Appendix 5: RNAHybrid analysis of psiCHECK-2 with OvHV-2-encoded miRNAs

RNAHybrids of ovhv2-miR-17-30 and ovhv2-miR-17-14 predicted to target firefly and renilla luciferase

```
target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -22.5 kcal/mol
p-value: 1.000000e+00

position  4070
target 5' C  CG  GU  UGUUCGUGGACG           G 3'
           GG  GA  GG            AGGUGCCCAAG
           UC  CU  CC            UCUGUGGGUUU
miRNA  3'    UA  GU                          5'


target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -20.4 kcal/mol
p-value: 1.000000e+00

position  3932
target 5' C   G    C  CC        C 3'
           GGA UGGC GG   UGCCCGA
           UCU ACUG CC   GUGGGUU
miRNA  3'          U  UCU       U 5'


target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -19.3 kcal/mol
p-value: 1.000000e+00

position  2945
target 5'  C       A   AGCUGCCUAU        A 3'
            GUG CAG AGA          CAUCCAGA
            UAC GUC UCU          GUGGGUUU
miRNA  3' UC   U   C                      5'
```

```
target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -18.4 kcal/mol
p-value: 1.000000e+00

position  2703
target 5' G     U U U          C 3'
           AGAUG C G   GCGCCUGG
           UCUAC G C   UGUGGGUU
miRNA  3'       U U CUC        U 5'


target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -17.3 kcal/mol
p-value: 1.000000e+00

position  3812
target 5' C  G    A CACUUCUUCAUCGU   C        G 3'
           GA GACG G              GGA CGCCUGAA
           CU CUGU C              UCU GUGGGUUU
miRNA  3' U  A    C                           5'


target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -15.0 kcal/mol
p-value: 1.000000e+00

position  3272
target 5'    C    UGUUCACCACCCU    C       U 3'
              GGCA             GG G UACCUGA
              CUGU             CC C GUGGGUU
miRNA  3' UCUA                   U U       U 5'
```

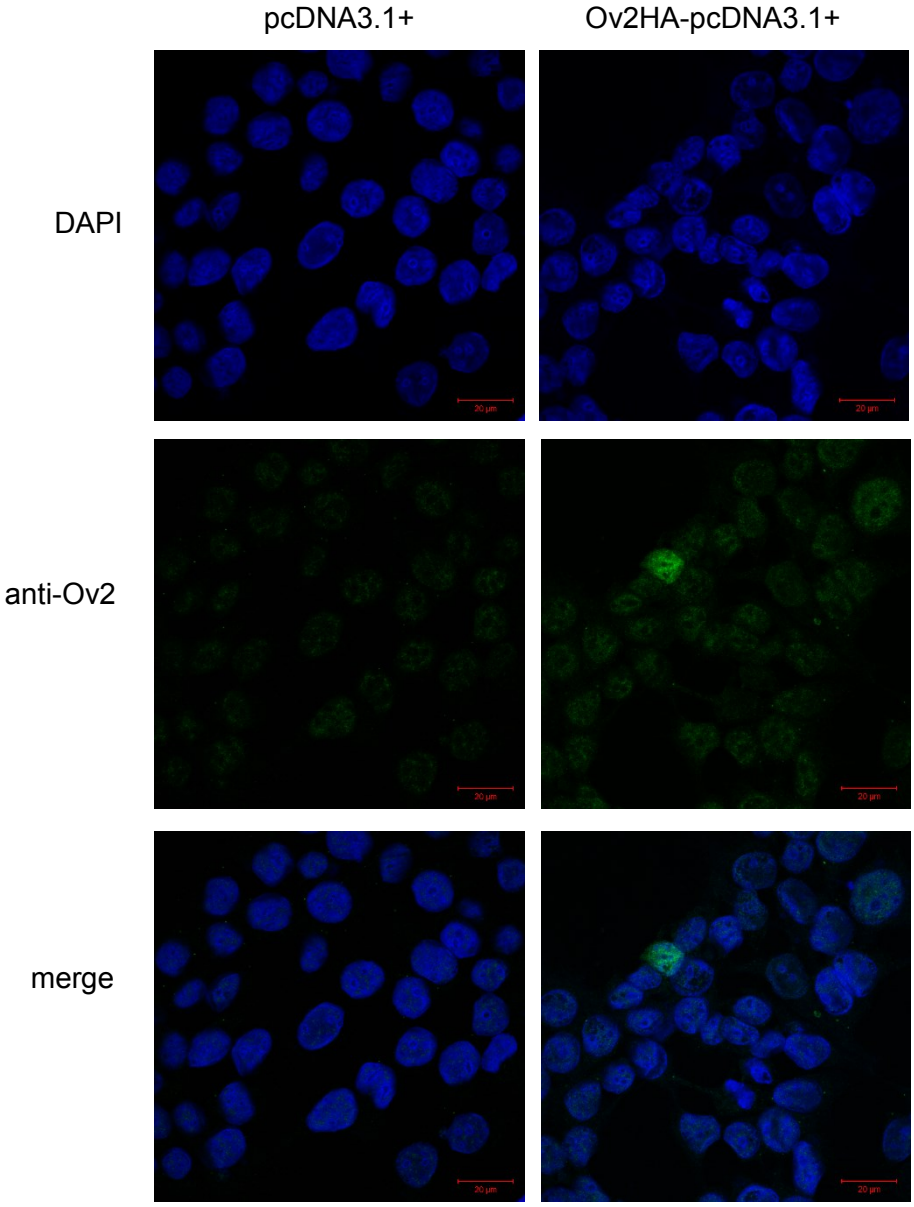
```
target: psiCHECK-2- Renilla
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -18.9 kcal/mol
p-value: 1.000000e+00

position  1299
target 5'      C U    C        G 3'
                C GGAG CAUUCAAG
                G CCUC GUGGGUUU
miRNA  3' UCUACU U    U          5'

target: psiCHECK-2- Renilla
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -16.9 kcal/mol
p-value: 1.000000e+00

position  1197
target 5' C  A  G  C    AAAUGG        A 3'
           GA GA GG GAGA      UGCUUGAG
           CU CU UC CUCU      GUGGGUUU
miRNA  3' U  A  G                      5'


target: psiCHECK-2- Renilla
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -16.8 kcal/mol
p-value: 1.000000e+00

position  1427
target 5'    G   C  C  C  UC        A 3'
              GGC AG GA GA  UGCCUAAG
              CUG UC CU CU  GUGGGUUU
miRNA  3' UCUA                      5'


target: psiCHECK-2- Renilla
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -16.8 kcal/mol
p-value: 1.000000e+00

position  1373
target 5'       A      CAAGCCCGACGU        U 3'
                 GGGAGG            CGUCCAGA
                 UCCUCU            GUGGGUUU
miRNA  3' UCUACUG                          5'
```

```
target: psiCHECK-2 - Renilla
length: 6273
miRNA : ovhv2-miR-17-30
length: 21

mfe: -15.0 kcal/mol
p-value: 1.000000e+00

position  1050
target 5'    C   U     GCU       C 3'
              GAC GGGGG   UGUCUGG
              CUG CCUCU   GUGGGUU
miRNA  3' UCUA   U               U 5'

target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-14
length: 25

mfe: -27.2 kcal/mol
p-value: 1.000000e+00

position  4068
target 5'  C       AGUGGU    G   ACG       C 3'
            GCGGCGG      GUUC UGG   AGGUGCC
            UGUUGUC      CGAG ACC   UUUACGG
miRNA  3' CU                 G             U 5'


target: psiCHECK-2 - firefly
length: 6273
miRNA : ovhv2-miR-17-14
length: 25

mfe: -19.6 kcal/mol
p-value: 1.000000e+00

position  2677
target 5' U     UCACCUAU  C A  A   C        U 3'
           GGACA        GC G GU CUU GAGAUGUC
           CUUGU        UG C CG GGA CUUUACGG
miRNA  3'                 U    A   C        U 5'


target: psiCHECK-2 - Renilla
length: 6273
miRNA : ovhv2-miR-17-14
length: 25

mfe: -22.9 kcal/mol
p-value: 1.000000e+00

position  1095
target 5' A     GAUCA    CAUCGUCCA G   A        U 3'
           GACAA     AGGC         U CUG GAGUGUCG
           UUGUU     UCCG         A GAC UUUACGGU
```

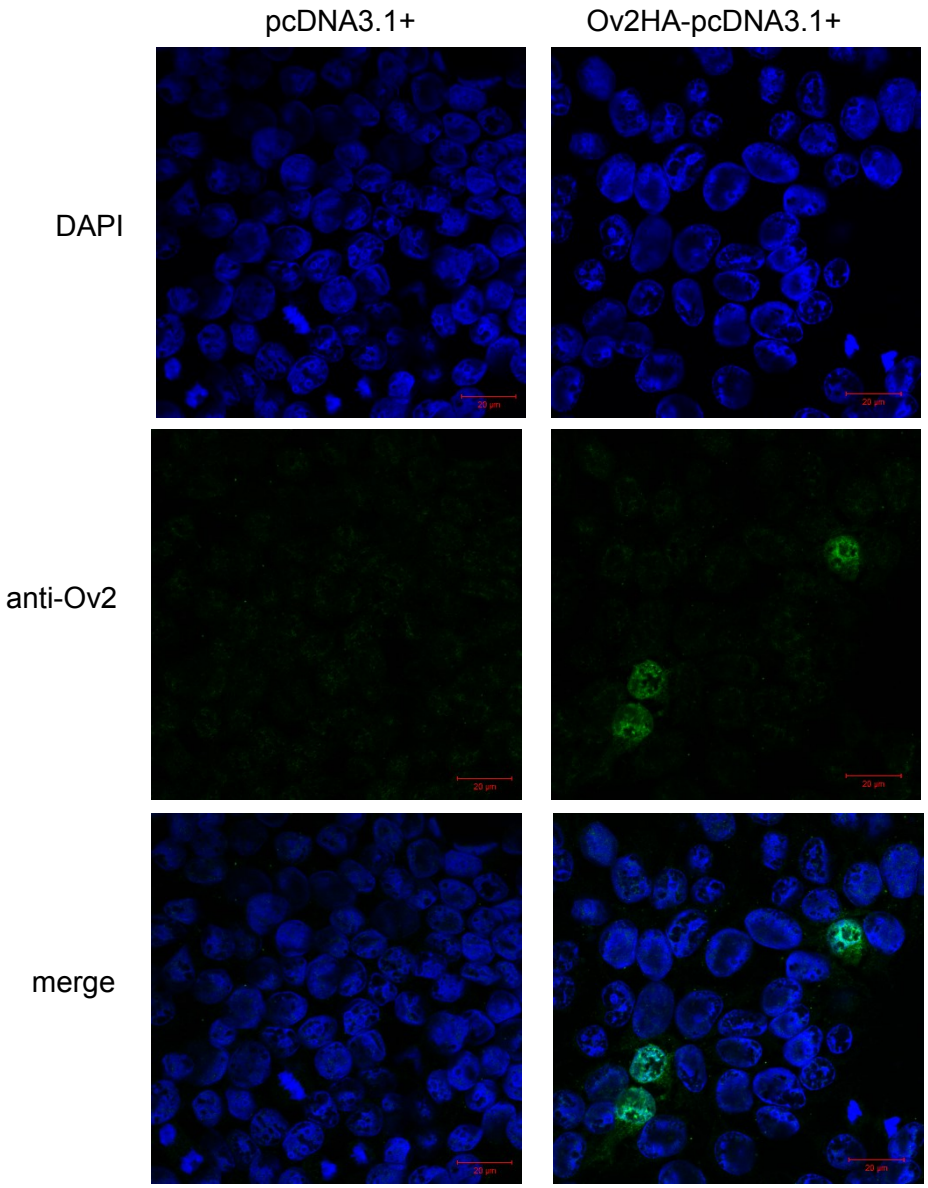
```
miRNA  3' C     G                  G   C          5'

target: psiCHECK-2 - Renilla
length: 6273
miRNA : ovhv2-miR-17-14
length: 25

mfe: -19.2 kcal/mol
p-value: 1.000000e+00

position  1431
target 5' C       AU  G   A        C 3'
           AGCGACG  CU CCU  AGAUGUU
           UUGUUGU  GA GGA  UUUACGG
miRNA  3' C       CC      CC       U 5'
```

Appendix 6: Immunofluorescence of pcDNA3.1+, Ov2HA-pcDNA3.1+ and Ov2-pcDNA3.1+ stained with an anti-Ov2 antibody and DAPI

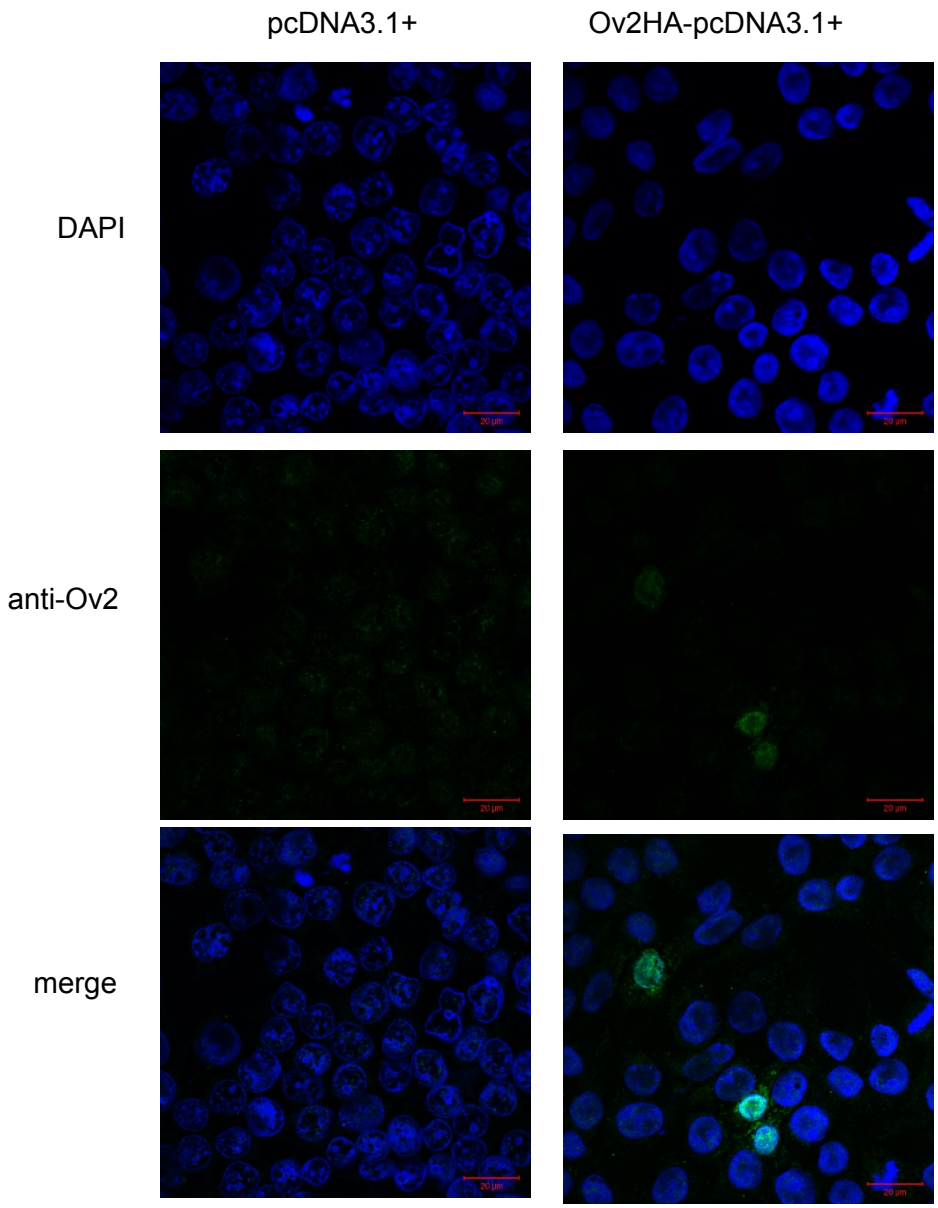
Immunofluorescence of pcDNA3.1+ and Ov2HA-pcDNA3.1+ at 4 hrs post transfection



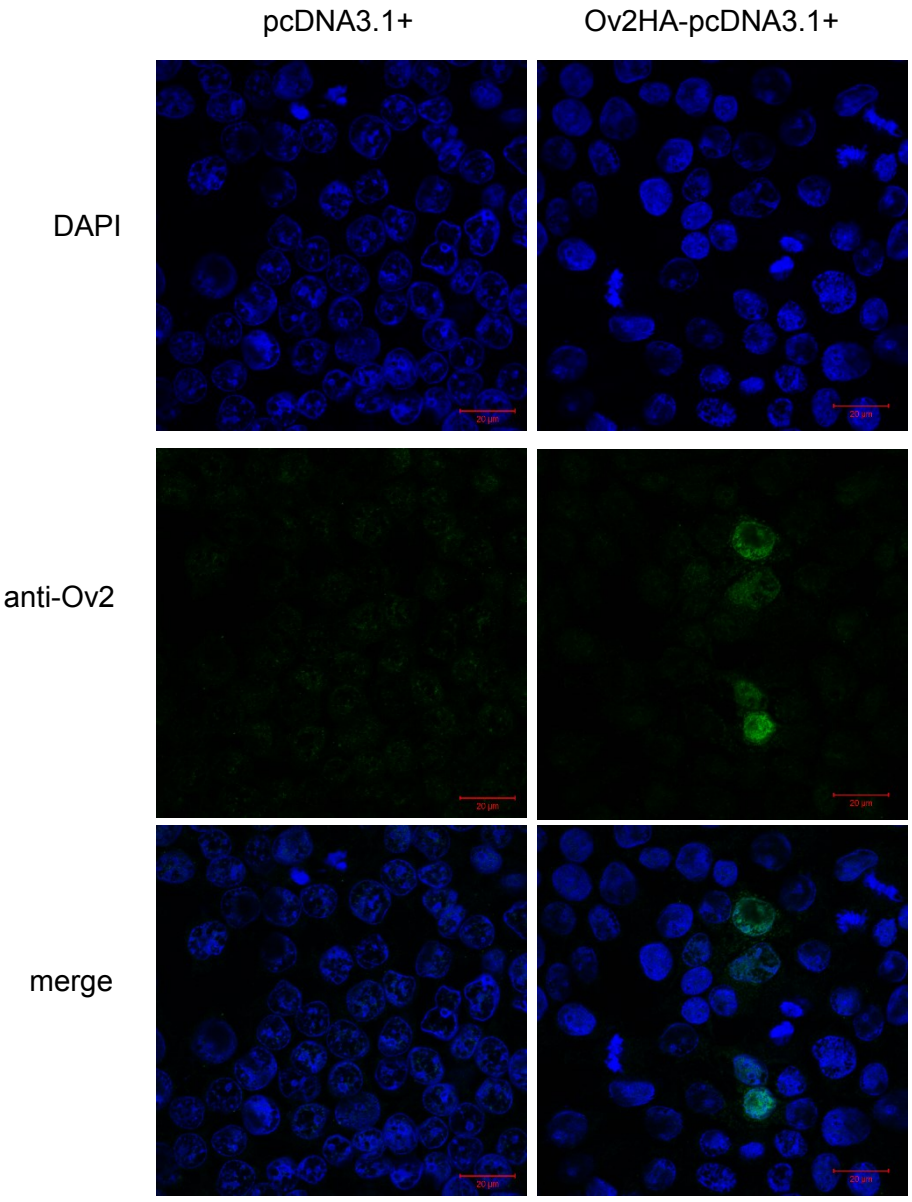
Immunofluorescence of pcDNA3.1+ and Ov2HA-pcDNA3.1+ at 8 hrs post transfection



Immunofluorescence of pcDNA3.1+ and Ov2HA-pcDNA3.1+ at 16 hrs post transfection



Immunofluorescence of pcDNA3.1+ and Ov2-pcDNA3.1+



Appendix 7: Proteins identified by Mass Spectrometry in pcDNA3.1+ and Ov2HA-pcDNA3.1+ immunoprecipitated samples

pcDNA3.1+ samples

Protein ID	Gene Name	Protein Score	No. unique peptides
M0R0F0	40S ribosomal protein S5 (Fragment) (RPS5)	934	11
P62829	60S ribosomal protein L23 (RPL23)	826	9
P0CW22	40S ribosomal protein S17-like (RPS17L)	652	9
P62269	40S ribosomal protein S18 (RPS18)	623	12
P62280	40S ribosomal protein S11 (RPS11)	358	7
P62701	40S ribosomal protein S4, X isoform (RPS4X)	356	8
P62081	40S ribosomal protein S7 (RPS7)	341	8
P10412	Histone H1.4 (HIST1H1E)	323	5
P62249	40S ribosomal protein S16 (RPS16)	317	8
P07437	Tubulin beta chain (TUBB)	231	8
P68363	Tubulin alpha-1B chain (TUBA1B)	230	5
Q9Y383	Putative RNA-binding protein Luc7-like 2 (LUC7L2)	223	4
P04350	Tubulin beta-4A chain (TUBB4A)	213	7
P62851	40S ribosomal protein S25 (RPS25)	209	7
P23396	40S ribosomal protein S3 (RPS3)	200	6
Q5VTE0	Putative elongation factor 1-alpha-like 3 (EEF1A1P5)	180	5
P62266	40S ribosomal protein S23 (RPS23)	179	6
P62273	40S ribosomal protein S29 (RPS29)	172	5
E1P5S2	RNA-binding protein 39 (RBM39)	164	6
P08107	Heat shock 70 kDa protein 1A/1B (HSPA1A)	162	4
P39019	40S ribosomal protein S19 (RPS19)	157	5
P10809	60 kDa heat shock protein, mitochondrial (HSPD1)	139	4
P06733	Alpha-enolase (ENO1)	134	6
E7ETK0	40S ribosomal protein S24 (RPS24)	124	6
P62913	60S ribosomal protein L11 (RPL11)	118	4
P62263	40S ribosomal protein S14 (RPS14)	108	3
H7BY10	60S ribosomal protein L23a (Fragment) (RPL23A)	106	3
E9PR30	40S ribosomal protein S30 (FAU)	104	3
P60709	Actin, cytoplasmic 1 (ACTB)	100	6

Q15365	Poly(rC)-binding protein 1 (PCBP1)	100	4
P14618	Pyruvate kinase PKM (PKM)	93	3
S4R435	Protein RPS10-NUDT3 (Fragment) (RPS10-NUDT3)	86	4
P58876	Histone H2B type 1-D (HIST1H2BD)	80	3
P60866	40S ribosomal protein S20 (RPS20)	78	3
P62854	40S ribosomal protein S26 (RPS26)	74	2
P62753	40S ribosomal protein S6 (RPS6)	69	2
E7EUT5	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	56	3
H0YMV8	40S ribosomal protein S27 (RPS27L)	52	2
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	52	2
Q06830	Peroxiredoxin-1 (PRDX1)	47	3
K7EMH1	60S ribosomal protein L22 (Fragment) (RPL22)	46	2
B7Z4C8	60S ribosomal protein L31 (RPL31)	43	2
X1WI28	60S ribosomal protein L10 (Fragment) (RPL10)	41	2

Ov2HA-pcDNA3.1+ samples

Protein ID	Gene Name	Protein Score	No. unique peptides
M0R0F0	40S ribosomal protein S5 (Fragment) (RPS5)	1227	10
P62829	60S ribosomal protein L23 (RPL23)	1043	8
P0CW22	40S ribosomal protein S17-like (RPS17L)	958	9
P62269	40S ribosomal protein S18 (RPS18)	925	13
P62701	40S ribosomal protein S4, X isoform (RPS4X)	757	8
P62280	40S ribosomal protein S11 (RPS11)	599	7
Q9Y383	Putative RNA-binding protein Luc7-like 2 (LUC7L2)	593	7
Q9NQ29	Putative RNA-binding protein Luc7-like 1 (LUC7L)	254	4
P10412	Histone H1.4 (HIST1H1E)	429	4
P68363	Tubulin alpha-1B chain (TUBA1B)	405	7
P62851	40S ribosomal protein S25 (RPS25)	373	7
P62081	40S ribosomal protein S7 (RPS7)	372	8
P39019	40S ribosomal protein S19 (RPS19)	371	5
P07437	Tubulin beta chain (TUBB)	358	10
P62249	40S ribosomal protein S16 (RPS16)	339	6
P10809	60 kDa heat shock protein, mitochondrial (HSPD1)	316	3
P06733	Alpha-enolase (ENO1)	315	6
P08107	Heat shock 70 kDa protein 1A/1B (HSPA1A)	296	10
E9PI65	Heat shock cognate 71 kDa protein (Fragment) (HSPA8)	106	3
P62266	40S ribosomal protein S23 (RPS23)	294	5
P62273	40S ribosomal protein S29 (RPS29)	292	4
P62854	40S ribosomal protein S26 (RPS26)	287	3
H7BY10	60S ribosomal protein L23a (Fragment) (RPL23A)	253	3
E9PR30	40S ribosomal protein S30 (FAU)	234	3
P23396	40S ribosomal protein S3 (RPS3)	233	7
P14618	Pyruvate kinase PKM (PKM)	206	3
P46776	60S ribosomal protein L27a (RPL27A)	199	5
P42766	60S ribosomal protein L35 (RPL35)	199	2

E7ETK0	40S ribosomal protein S24 (RPS24)	198	5
Q06830	Peroxiredoxin-1 (PRDX1)	185	3
P62937	Peptidyl-prolyl cis-trans isomerase A (PPIA)	181	5
P08238	Heat shock protein HSP 90-beta (HSP90AB1)	142	4
P07900	Heat shock protein HSP 90-alpha (HSP90AA1)	57	2
P62263	40S ribosomal protein S14 (RPS14)	136	4
Q14498	RNA-binding protein 39 (RBM39)	130	5
C9J4Z3	60S ribosomal protein L37a (RPL37A)	123	2
P07195	L-lactate dehydrogenase B chain (LDHB)	121	2
S4R435	Protein RPS10-NUDT3 (Fragment) (RPS10-NUDT3)	120	5
H0Y5B4	60S ribosomal protein L36a (Fragment) (RPL36A)	115	2
A2A3R5	40S ribosomal protein S6 (RPS6)	105	2
Q15365	Poly(rC)-binding protein 1 (PCBP1)	100	4
F5H4F9	40S ribosomal protein S3a (RPS3A)	99	3
P60866	40S ribosomal protein S20 (RPS20)	86	3
P62913	60S ribosomal protein L11 (RPL11)	82	3
P60709	Actin, cytoplasmic 1 (ACTB)	75	5
J3KPD9	Nucleoside diphosphate kinase B (NME2)	74	2
B7Z4C8	60S ribosomal protein L31 (RPL31)	73	2
E7EUT5	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	71	3
P50990	T-complex protein 1 subunit theta (CCT8)	62	2
P62241	40S ribosomal protein S8 (RPS8)	60	2
C9J9K3	40S ribosomal protein SA (Fragment) (RPSA)	57	2
Q2VSN7	Ov2 protein	55	2
X1WI28	60S ribosomal protein L10 (Fragment) (RPL10)	48	3

Appendix 8: Proteins Identified by Mass Spectrometry with only one unique peptide

pcDNA3.1+ samples

Protein ID	Gene Name	Protein Score
P42766	60S ribosomal protein L35 (RPL35)	142
C9J4Z3	60S ribosomal protein L37a (RPL37A)	106
P62857	40S ribosomal protein S28 (RPS28)	97
H0Y8L7	40S ribosomal protein S3a (Fragment) (RPS3A)	96
H0YH80	Heterogeneous nuclear ribonucleoprotein A1 (Fragment) (HNRNPA1)	70
J3KNN5	Probable ATP-dependent RNA helicase DDX41 (Fragment) (DDX41)	68
G3V461	Creatine kinase B-type (Fragment) (CKB)	64
C9JNW5	60S ribosomal protein L24 (RPL24)	62
P07195	L-lactate dehydrogenase B chain (LDHB)	55
A6NE09	40S ribosomal protein SA (RPSAP58)	54
P37108	Signal recognition particle 14 kDa protein (SRP14)	54
P62979	Ubiquitin-40S ribosomal protein S27a (RPS27A)	53
E9PN89	Heat shock cognate 71 kDa protein (Fragment) (HSPA8)	50
D3YTB1	60S ribosomal protein L32 (Fragment) (RPL32)	49
F5GZK5	T-complex protein 1 subunit eta (CCT7)	49
E9PLM6	Midkine (MDK)	44

Ov2HA-pcDNA3.1+ samples

Protein ID	Gene Name	Protein Score
P62857	40S ribosomal protein S28 (RPS28)	140
H0YLA2	Signal recognition particle 14 kDa protein (SRP14)	108
C9JNW5	60S ribosomal protein L24 (RPL24)	91
E9PLD3	Uncharacterized protein OS=Homo sapiens)	91
P18669	Phosphoglycerate mutase 1 (PGAM1)	84
B7Z2F4	T-complex protein 1 subunit delta (CCT4)	80
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1)	79
M0R1M6	Ubiquitin-60S ribosomal protein L40 (Fragment) (UBA52)	76
U3KQK0	Histone H2B (HIST1H2BN)	72
G3V461	Creatine kinase B-type (Fragment) (CKB)	67
K7EJ44	Profilin 1, isoform CRA_b (PFN1)	62
P62979	Ubiquitin-40S ribosomal protein S27a (RPS27A)	62
J3KPP4	Cisplatin resistance-associated overexpressed protein, isoform CRA_b (LUC7L3)	57
H0YH80	Heterogeneous nuclear ribonucleoprotein A1 (Fragment) (HNRNPA1)	51
H7BY16	Nucleolin (Fragment) (NCL)	51
K7ELC2	40S ribosomal protein S15 (RPS15)	48
E7EQG2	Eukaryotic initiation factor 4A-II (EIF4A2)	48
P62244	40S ribosomal protein S15a (RPS15A)	47
Q5T6W1	Heterogeneous nuclear ribonucleoprotein K (HNRNPK)	47
K7EJT5	60S ribosomal protein L22 (Fragment) (RPL22)	41

Appendix 9: RNAHybrid analysis of the 3'UTR of Ov2

MIRNA : ovhv2-miR-17-23
length: 21

mfe: -19.3 kcal/mol
p-value: undefined

position 820
```
target 5'   A  AAAAAGG         C 3'
             GG       CAGUGUGUA
             UC       GUCACACAU
miRNA  3' AGA  GAGAAA          A 5'
```

MIRNA : ovhv2-miR-17-17
length: 22

mfe: -23.7 kcal/mol
p-value: undefined

position 977
```
target 5'     G      ACUUUUUAUCC          A 3'
               GUUGCU           UGGGGAUCCU
               CAGUGA           AUUCCUAGGA
miRNA  3' GCAAG                           A 5'
```

MIRNA : ovhv2-miR-17-13
length: 23

mfe: -24.3 kcal/mol
p-value: undefined

position 1072
```
target 5' A   U  GAGGGGA     GAUAG        A 3'
           ACC CG       UC CA     GGACCCAA
           UGG GC       AG GU     CCUGGGUU
miRNA  3'     C           A  ACAA          5'
```

MIRNA : ovhv2-miR-17-13
length: 23

mfe: -21.3 kcal/mol
p-value: undefined

position 1104
```
target 5'              C         G 3'
                        UGGACCCAA
                        ACCUGGGUU
miRNA  3' UGGCGCAGAGUACA          5'
```

Appendix 10: RNAHybrid analysis of ovhv2-miR-17-10 against Ov2 allowing G:U pairing

RNAHybrid analysis against the mutated Ov2 sequence

```
MIRNA : ovhv2-miR-17-10
length: 23

mfe: -14.9 kcal/mol
p-value: undefined

position  93
target 5'    A       UCAC            A 3'
              AGG GCA    GC   AAUUUUA
              UCC CGU    CG   UUGAAGU
miRNA  3' UAGG   A         ACA         5'
```

RNAHybrid analysis against the Ov2 sequence allowing for G:U pairing and perfect complementarity between nucleotides 1 and 7 or nucleotides 2 and 8

```
MIRNA : ovhv2-miR-17-10
length: 23

mfe: -15.0 kcal/mol
p-value: undefined

position  285
target 5' U     UAGUG     CAUG GGGAGAAGUUACAAA       U 3'
           UCUAG     GCGGU    G               UGAUUUU
           AGGUC     CGUCG    C               AUUGAAG
miRNA  3' U     CA        A                         U 5'


MIRNA : ovhv2-miR-17-10
length: 23

mfe: -17.5 kcal/mol
p-value: undefined

position  454
target 5' U  A    AGAAG     GAACA       G 3'
           UC CAGG     GCAGU     GGUUUUG
           AG GUCC     CGUCG     UUGAAGU
miRNA  3' U       A         ACA           5'


MIRNA : ovhv2-miR-61-1
length: 25

mfe: -19.7 kcal/mol
p-value: undefined

position  477
target 5'  G        AAG   UA        A 3'
            GUU UUGG   AGC   UUCCCAG
            CAG AGUC   UCG   AGGGGUU
miRNA  3' UG   C    GG    UGC         5'
```

Appendix 11: Publication

OPEN ACCESS Freely available online PLOS ONE

Expression of Ovine Herpesvirus -2 Encoded MicroRNAs in an Immortalised Bovine – Cell Line

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Abstract

Ovine herpesvirus-2 (OvHV-2) infects most sheep, where it establishes an asymptomatic, latent infection. Infection of susceptible hosts e.g. cattle and deer results in malignant catarrhal fever, a fatal lymphoproliferative disease characterised by uncontrolled lymphocyte proliferation and non MHC restricted cytotoxicity. The same cell populations are infected in both cattle and sheep but only in cattle does virus infection cause dysregulation of cell function leading to disease. The mechanism by which OvHV-2 induces this uncontrolled proliferation is unknown. A number of herpesviruses have been shown to encode microRNAs (miRNAs) that have roles in control of both viral and cellular gene expression. We hypothesised that OvHV-2 encodes miRNAs and that these play a role in pathogenesis. Analysis of massively parallel sequencing data from an OvHV-2 persistently-infected bovine lymphoid cell line (BJ1035) identified forty-five possible virus-encoded miRNAs. We previously confirmed the expression of eight OvHV-2 miRNAs by northern hybridization. In this study we used RT-PCR to confirm the expression of an additional twenty-seven OvHV-2-encoded miRNAs. All thirty-five OvHV-2 miRNAs are expressed from the same virus genome strand and the majority (30) are encoded in an approximately 9 kb region that contains no predicted virus open reading frames. Future identification of the cellular and virus targets of these miRNAs will inform our understanding of MCF pathogenesis.

Citation: Nightingale K, Levy CS, Hopkins J, Grey F, Esper S, et al. (2014) Expression of Ovine Herpesvirus -2 Encoded MicroRNAs in an Immortalised Bovine – Cell Line. PLoS ONE 9(5): e97765. doi:10.1371/journal.pone.0097765

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Introduction

Malignant catarrhal fever (MCF) is a fatal disease of cattle, deer and pigs caused by one of two related gammaherpesviruses (γ-HV), ovine herpesvirus -2 (OvHV-2) or alcelaphine herpesvirus 1 (AlHV-1). MCF is characterized by sudden onset of fever followed by lymphadenopathy, leukocytosis, severe congestion, necrosis and erosion of the oral, conjuctival and nasal mucosae [1]. The disease occurs as the result of infection of susceptible hosts by contact with an asymptomatic carrier species that acts as a virus reservoir. OvHV-2 is the major cause of sheep associated MCF worldwide [1]. It infects most sheep, where it establishes a latent but asymptomatic infection. AlHV-1 is the major cause of MCF in sub-Saharan Africa where the wildebeest is the asymptomatic carrier species.

The same cell populations are infected in both cattle and sheep [2,3] but only in cattle does virus infection cause dysregulation of cell function leading to uncontrolled proliferation, cytotoxicity and disease. In both species the infected cells arise from common lymphoid progenitors [4] but interleukin 2-dependent cell lines can be cultured only from affected animals. There is currently no tissue culture system for OvHV-2 and such cell lines are the only resource for working with the virus. In affected species the infected cells have been described as large granular lymphocytes (LGLs) [5]. The immortalized cell lines have a variety of phenotypes; all express CD2 but vary in their expression of CD4 and CD8 [2,3].

The mechanism by which OvHV-2 induces MCF is unknown; virus-induced cytopathology is thought not to be involved in lesion development and it has been proposed that tissue damage arises from non-antigen specific, MHC unrestricted cytotoxicity of the LGLs. The key question in understanding OvHV-2 pathogenesis is; why infection of the same cell type in two closely related species leads to such different disease outcomes, i.e. lymphoid cell dysregulation and MCF in cattle versus asymptomatic infection in sheep.

MicroRNAs (miRNAs) constitute a large family of small, non-coding RNAs functioning in post-transcriptional regulation of mRNA in eukaryotes [6–8] as well as in a number of viruses, particularly in the members of the family *Herpesviridae* [9–11]. miRNA regulation of expression is by binding of the miRNA seed sequence (~nucleotides 2–8) to complementary sequences in target mRNAs and directing these for degradation or translational silencing; the majority of miRNAs target sequences within the 3′ UTR [6]. Herpesvirus-encoded miRNAs have been shown to be effective regulators of both cellular and virus gene expression and to influence cell processes including the cell cycle [12–17]. The pathology of the herpesviruses Epstein Barr virus (EBV) and Marek's disease virus (MDV) also involves aberrant lymphocyte proliferation, and virus-encoded miRNAs play a key role in the induction of this proliferation. Furthermore, deletion of a single (MDV) or a small cluster (EBV) of virus-encoded miRNAs

Table 1. OvHV-2-encoded microRNAs.

ovhv2-miR-	Previous name	Abundance RNA-seq	Walz *et al.*	5'nt	3'nt	Sequence	Validation Method
Ov2-2	miR-1	10588	√	927	906	AAGGCUUGAUAAGUAGCACUGA	Levy *et al.*
Ov2-1		253	√	1182	1161	AUGCUUGUUUUAGGCCCCAUGAA	Sequencing
17-30		484	√	27722	27702	UUUGGGUGUUCUCCUGUCAUCU	Sequencing
17-29	miR-2	39169	√	27903	27881	AUCUUGGAGGCAUCUGUCAGUAG	Levy *et al.*
17-28		6200	√	28066	28045	UCUAGGUUGCAUUUUGCUGUAG	Sequencing
17-27		6015	√	28209	28188	CCCACAUUUAAGGUGCUCGUGU	Sequencing
17-26		322	√	28381	28361	AUAUUGCGUUUUAGACGCAAGUA	Sequencing
17-25		4717	√	28515	28495	CAAUGCUGCUUUGGUGCCUCA	Sequencing
17-24		1014	√	28683	28661	GGGUUCCUCGGAGUGGAUAUUGUU	Sequencing
17-23		21686	√	28781	28761	AUACACACUGAAAGAGGUAGA	Sequencing
17-22		740	√	28888	28866	AUAAGGCCAACACUAGGUGCUGU	Sequencing
17-21		31227	√	29052	29028	AAGCACCUUGGGUGAUGUCUCUGUU	Sequencing
17-20	miR-3	14694	√	29248	29226	UCUGUAUCAUAGGGGUUGUGUUG	Levy *et al.*
17-19		6487	√	29345	29323	AAGCAUAGCUGGGAGUGUCUAGA	Specific cDNA
17-18		9219	√	29455	29434	UAGUAGUCCGUUAACGCAAAGU	Sequencing
17-17	miR-4	17508	√	29637	29616	AAGGAUCCUUAAGUGACGAACG	Levy *et al.*
17-16		8095	√	29745	29725	UAAACUGGUGGUAGGCGGUCU	Sequencing
17-15		2967	√	29945	29923	UAGCAGUUAUGCAGGUAUCUGGU	Sequencing
17-14		3794	√	30091	30067	UGGCAUUUCCAGGACCUGUUGUUC	Sequencing
17-13		24497	√	30338	30316	UUGGUCCAACAUGAGAGCGGGU	Sequencing
17-12		14966	√	30510	30489	UAUGUCAGAAGUGAAGCUGAGA	Specific cDNA
17-11		1357	√	30632	30611	UGGUUUGCAUCUGCACCCAGUU	Sequencing
17-10	miR-5	11187	√	30818	30796	UGAAGUUACAGCUGGCACCUGGAU	Levy *et al.*
17-9		5457	√	30960	30936	UAGAGUUACUAAGGAUUCCCUGGUA	Sequencing
17-8		942	√	31085	31066	AAUCGCCGGUGGCCUCUAG	Sequencing
17-7		351	√	31359	31340	UAUAGACGGGUAUGCUGCCG	Specific cDNA
17-6	miR-6	10378	√	31482	31440	UAUUUUUUAGCGGAGACCUCUAGG	Levy *et al.*
17-5		1535	√	31603	31582	CCUUUUUGGUGAGAUUGCCCUGU	Sequencing
17-4	miR-7	517	√	32075	32054	GAUUUGAUAAAGCCUGCCUGCG	Sequencing
17-3		16574	√	36313	36289	GAAGGCGCAUCAUAGACACCACUC	Levy *et al.*
17-2		7834	√	36482	36461	ACCCCGGGGGUAUGUGCAGGAC	Sequencing
17-1	miR-8	46048	√	36575	36554	UGCCUCAGCGUGACUGCUCUUC	Levy *et al.*
24-1		1	x	48585	48560	GAGCAGUAGUACACAGCAGACAACAU	Sequencing

Table 1. Cont.

ovhv2-miR-	Previous name	Abundance RNA-seq	Walz *et al.*	5' nt	3' nt	Sequence	Validation Method
61-1		1	x	96435	96411	UUGGGGACGUGCUGGCUGACGCGU	Specific cDNA
73-1		6064	x	117122	117100	UAAUCUCUGCUCCAAUGUAAAU	Specific cDNA

The 35 validated miRNAs are listed. **Previous name**: designation in Levy *et al.* 2012. **Abundance (RNA-seq)**: Total number of sequence tags representing the miRNA in the RNA-seq data [22] **Walz *et al.***: miRNAs predicted by Walz *et al.*, J = yes; X = no. **5' nt/3' nt**: the first and last nucleotides of the mature miRNA on the OvHV-2 genome (AY839795) [28]. **Sequence**: sequence of the mature miRNA. **Validation method**: Levy *et al.*: validated previously [22]; Sequencing: validated using the RT-PCR and subsequent sequencing; Specific cDNA: validated using the approach of Vaníková-Gatic *et al.* [24].

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attenuates these viruses [18–20]. Consequently we hypothesized that OvHV-2 encodes miRNAs that target host mRNAs and that these constrain virus pathology in sheep and/or induce MCF pathology in cattle. Identification and characterization of OvHV-2-encoded miRNAs is therefore essential to allow this hypothesis to be addressed.

In a previous study we reported the results of massively parallel sequencing of small RNAs present in the BJ1035 OvHV-2 immortalized bovine LGL cell line and predicted that the virus encodes up to forty-five miRNAs [21]. Eight of these were confirmed by northern hybridization [22]. Here we extend these studies using two PCR protocols to investigate the expression of the other predicted OvHV-2 miRNAs.

Methods

Cell culture

BJ1035 cells, an immortalized bovine T cell line from an animal naturally infected with OvHV-2 [3], was grown in suspension culture in Iscove's Modified Dulbecco's Medium (Invitrogen, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (Sera Laboratories International, Haywards Heath, UK), 1% (v/v) penicillin-streptomycin (Invitrogen), 20 U/ml interleukin 2 (Novartis Pharmaceuticals UK., Camberley, UK) and incubated at 37°C, 5% CO_2. Bovine lymphoid cells were isolated from fresh blood and lymphoblasts generated as described previously [23]. All relevant procedures were approved by the University of Edinburgh Ethical Review Committee and carried out under an Animal (Scientific Procedures) Act 1996 project licence.

Reverse Transcription PCR (RT-PCR)

Bovine lymphoblasts and BJ1035 cells were pelleted by centrifugation and total RNA extracted using Trizol (Invitrogen). cDNA was generated using the miScript II RT Kit (Qiagen, Crawley, UK) that uses oligo-dT to prime reverse transcription. For each predicted miRNA, a specific forward primer spanning the first 15–16 nucleotides of the miRNA was designed (Table S1) allowing subsequent sequence confirmation of the identity of the amplified miRNA RT-PCR was performed using miScript SYBR green PCR kit (Qiagen), with the supplied universal reverse primer and the miRNA specific forward primers using the annealing temperatures shown in Table S1. PCR products were fractionated by electrophoresis using 3% agarose, purified using the Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, Amersham, UK) and cloned using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA was isolated using the Qiaprep Spin Miniprep Kit (Qiagen), analysed by restriction enzyme digestion and sequenced (GATC Biotech, London UK).

miRNA specific reverse transcription

Individual miRNA specific primers (Table S2) were used to prime for reverse transcriptase. Each primer also has a 5' conserved region recognised by a universal reverse primer (5'-GTGCAGGGTCCGAGGT-3') [24]. An initial reaction to form the hairpin structure of the reverse transcription primer (200 μM) was carried out using the following conditions: 95°C for 30 min, 72°C for 2 min, 37°C for 2 min then 25°C for 2 min. The concentration of the reverse transcription primer after annealing was 50 μM. For the reverse transcription reaction 5 μM reverse transcription primer was used to prime 100 ng of RNA. Conditions for the reverse transcription reaction were as follows: 16°C for 30 min, 42°C for 30 min then 85°C for 5 min. PCR was thenperformed using miRNA specific forward primers (Table S2) and the following conditions: 5 min at 95°C, 40 cycles of: 30 s at

```
                    A    GGC  ACUGUG
miR-216a       5' UAAUCUC GCU    A      A 3'
ovhv2-miR-73-1 5' UAAUCUC GCU    A      A 3'
                    U    CCA  UUGUAA   U
```

Figure 1. Comparison of the sequences of ovhv2-miR-73-1 and miR-216a. Seed sequences (nt 1-7) are shown in red.
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95°C, 30 s at 60°C, 60 s at 72°C; then finally 7 min at 72°C. PCR products were fractionated by electrophoresis using 3% agarose. Reactions were performed in triplicate. The presence of a product in the BJ1035 cells but not in the uninfected bovine lymphoblast cells was taken as proof of OvHV-2 miRNA expression.

Results

Using northern hybridization we have previously demonstrated the expression of the eight of the 45 predicted miRNAs that were represented by the highest number of reads in our parallel sequencing data [22]. RT-PCR using miRNA specific forward primers and a universal reverse primer for the remaining 37 predicted miRNAs initially identified 22 virus-encoded miRNAs (Table S1, Table 1).

To investigate if the remaining 15 miRNAs are expressed, an alternative miRNA specific reverse transcription PCR strategy was adopted. The expression of a predicted miRNA (ovhv2-miR-73-1) that was shown to have seed sequence homology but no 3' sequence homology with mammalian miR-216a (Figure 1) was also analysed by this method. The presence of a product in the BJ1035 cells but not in the uninfected bovine lymphoblast cells was taken as proof of OvHV-2 miRNA expression. As the forward primer ends at the nucleotide adjacent to that from which cDNA is primed, sequencing of the product would generate only primer sequence. To confirm that amplification was from miRNA and not genomic DNA a no RT control was performed and no amplification was observed with any primer set. Expression of a further 5 miRNAs, including ovhv2-miR-73-1, was confirmed, (Figure 2). Figure 3 shows the location and direction of transcription of the thirty-five validated OvHV-2-encoded miRNAs.

Discussion

The number of miRNAs expressed by individual herpesviruses ranges from 8 to 50 [25] and here we demonstrate that OvHV-2 encodes 35 miRNAs. Using a predictive algorithm Walz *et al.* [26] predicted 61 hairpin sites in OvHV-2 that might encode miRNAs; 32 of which we have now confirmed to be expressed (Figure 3, Table 1), three of our validated miRNAs were not predicted by Walz *et al.*. All of the validated miRNAs expressed by OvHV-2 are transcribed in the same orientation, right to left. By convention virus-encoded miRNAs are named in relation to the nearest open reading frame (ORF) transcribed in the same direction as the miRNA; we have followed this convention in naming the OvHV-2-encoded miRNAs. Those identified in Levy *et al.* and discussed in Riaz *et al.*[22,27] have been renamed to adhere to this nomenclature (Table 1). Two miRNAs are encoded at the left hand end of the genome in the region between the terminal repeat and the 3' end of ORF Ov2, these have been named ovhv2-miR-Ov2-1 and ovhv2-miR-Ov2-2 (ovhv2-miR-1 in Levy *et al.* 2012[22]). The OvHV-2 genome, like that of AlHV-1 and equine herpesvirus 2 (EHV-2) contains two large regions of the genome with no predicted open reading frames [28]. The majority (30) of the identified miRNAs are encoded as two clusters at either end of the larger of these two regions. One cluster, spanning 4377 bp at the left end of this region, contains 27 miRNAs; a smaller cluster of three miRNAs is encoded in a 320 bp region at the right hand end of this region (Figure 3). These miRNAs have been named ovhv2-miR-17-1 to -30. Ovhv2-mir-17- 29, -20, -17, -10, -6, -3 and -1 were previously designated ovhv2-miR-2 to -8. One miRNA is encoded in the non-coding region situated toward the right end of the genome and is designated ovhv2-miR-73-1. The remaining miRNAs are encoded within ORFs 24 and 61 and are designated ovhv2-miR-24-1 and ovhv2-miR-61-1.

ovhv2-miR-73-1 was the only OvHV-2-encoded miRNA to show seed sequence homology to any other reported miRNA, miR-216a (Figure 1). This miRNA is broadly conserved in vertebrates and targets *PTEN* and *YBX1* [29,30] down regulation of which can lead to increased cell survival, hypertrophy, sclerosis, and a decreased cellular response to stress [31], all symptoms observed in MCF. We are currently investigating the cellular genes targeted by ovhv2-miR-73-1.

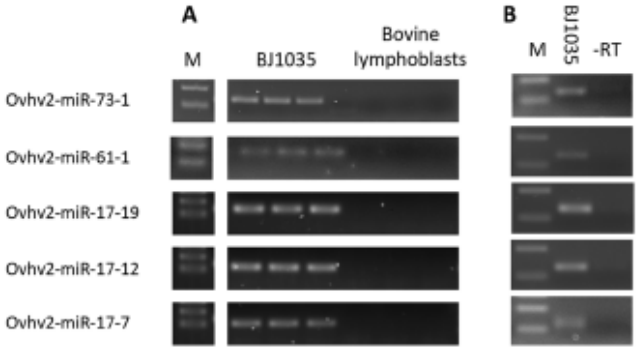

Figure 2. Analysis of OvHV-2-encoded miRNA expression using miRNA specific RT-PCR. A) Expression of five predicted OvHV-2-encoded miRNAs in BJ1035 cells but not uninfected bovine lymphoblasts was confirmed by miRNA specific RT-PCR. Each assay was carried out in triplicate. M: 50 bp and 100 bp markers B) miRNA specific RT-PCR was carried out along with an no RT (-RT) control.
doi:10.1371/journal.pone.0097765.g002

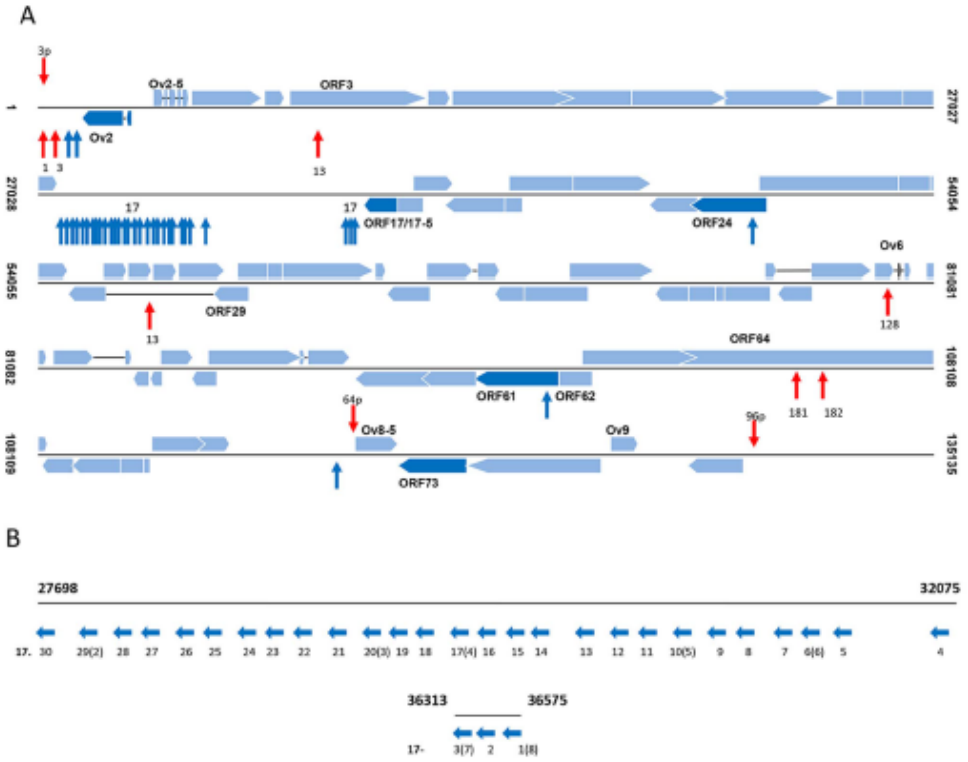

Figure 3. Location of miRNAs in the OvHV-2 genome. A) The relative positions of the predicted miRNAs in the OvHV-2 genome are shown. Numbering is from Hart *et al.*[28]. The genome is represented by a thin line and open reading frames (ORFs) are indicated by blue boxes. Boxes above the line represent ORFs transcribed left to right; those below the line represent ORFs which are transcribed right to left. Only those ORFs adjacent to predicted miRNAs are named. Arrows indicate the position of the predicted miRNAs; arrows above the line represent miRNAs transcribed left to right, those below the line represent miRNAs which are transcribed right to left. Dark blue vertical arrows indicate validated miRNAs. Red arrows indicate non-validated miRNAs named according to the groups listed in Table 1. Those OvHV-2 ORFs closest to validated miRNAs and after which those miRNAs are named are shown in darkblue. B) The locations of ovhv2-miR-17-1 to -3 0 are shown in more detail.
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AlHV-1 is the causative agent of wildebeest-associated MCF and the sequence identity between the individual ORFs of OvHV-2 and AlHV-1 varies from 22–83% ([28]). No significant sequence similarity was found between the non-coding regions of the two viruses and Blastn analysis failed to identify miRNAs in the AlHV-1 genome with sequence homology to any OvHV-2 miRNA. A lack of conservation of miRNAs between closely-related viruses has also been observed in different Marek's disease virus strains [32].

Herpesviruses are generally host species specific and are considered to have co-evolved with their natural host [33]. Herpesvirus-encoded miRNAs have been shown to regulate both virus and host gene expression [12–17] and it is likely that these miRNAs have also co-evolved with their host targets. The different disease outcomes seen in sheep (natural, co-evolved host) and cattle ("foreign" host may be the result of different virus:host interactions. Our hypothesis is that OvHV-2-miRNAs interact differently with sheep and cattle genes and that these differences play a role in the differing disease outcomes. It is likely that AlHV-1 does encode miRNAs, but that they have co-evolved to target wildebeest (natural host) genes in a similar manner to which OvHV-2 miRNAs have evolved to target sheep genes.

The identification of the miRNAs encoded by OvHV-2 allows us to study how these miRNAs affect host and virus gene expression. We have recently shown that viral genes ORF20 (cell cycle inhibition), ORF 50 (reactivation) and ORF 73 (latency maintenance) are targeted by ovhv-miR-17-29; ovhv2-miR-17-10 and ovhv2-miR-17-1 respectively[27]. The identification of host targets of the 35 OvHV2-miRNAs is not straightforward. Bioinformatic analysis of potential targets for the OvHV2-miRNAs in the sheep and cattle genome resulted in the identification of more than 100,000 possible targets in each genome, an unrealistic number to investigate. We are current

using experimental approaches to investigate cellular targets of the OvHV-2-miRNAs.

Supporting Information

Table S1 Sequences of specific forward PCR primers and related annealing temperatures. The sequence of the forward primers and annealing temperatures used to analyse expression of the predicted ovhv2-miRNAs are shown. Those miRNAs which were successfully validated using approach are shown in bold. "Group 1" etc. represent predicted miRNAs which were not shown to be expressed.
(DOCX)

Table S2 Sequence of miRNA specific Reverse transcription primers and specific 5' forward primers. For each of the miRNAs which were not validated using the miScript assay, the sequence of the primers used to prime cDNA synthesis, the specific 5' forward primers and the sequence of the universal reverse PCR primer are shown. "Group 1" etc. represent predicted miRNAs which were not shown to be expressed.
(DOCX)

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Author Contributions

Conceived and designed the experiments: KN RGD JH FG. Performed the experiments: KN CSL SE. Analyzed the data: KN JH FG RGD. Wrote the paper: RGD.

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